VIRUS DISEASES OF MAN

by

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Dedicated
to Our Friend
BERNARD HALLEY STEWART
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President
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AUTHORS' PREFACE TO THE FIRST EDITION

A NOTICEABLE feature of preser
taken in virus infections, and
an up-to-date account of the
proved to be the cause of human disease.

Our primary objective has been to provide a useful reference volume for the medical laboratory worker whether engaged in research or academic teaching, or confronted with the diagnosis of suspected cases of virus infection. At the same
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diagnosis,

resent may prove of real help to those members of the medical profession entrusted with safeguarding the health of His Majesty's Forces serving in different parts of the world, where specific virus diseases are endemic.

The demand upon our space for the inclusion of clinical data has compelled us to omit from this volume a certain amount of technical matter mainly of academic interest, but we have given in the text numerous references in order that those interested in such subjects may be able to refer to original papers. Throughout the book, the abbreviations adopted for the titles of journals cited in the reference columns conform to the contracted format recommended in the *World List of Scientific Periodicals*,[1] and we trust that the extensive bibliography will be of assistance to workers situated at a distance from libraries.

In this monograph we have not attempted strictly to classify virus infections, but we have grouped the various diseases according to the system mainly involved, in the following arbitrary order: skin diseases, infective fevers, tropical diseases, respiratory diseases, eye diseases, and diseases of the nervous system.

As a general rule, it will be found that the actual length of text devoted to any one disease depends more on the amount of relevant literature published than on the precise intrinsic importance of the particular subject. Where a certain virus is one which predominantly infects animals, and man but occasionally (e.g., foot-and-mouth disease and pseudorabies), its characteristics are only briefly described.

Viruses peculiar to animals or plants are not discussed, and likewise we have not dealt with the Rickettsiae, since our purview has been expressly limited to an account of only those diseases of man in which the causal agent has been definitely shown to come within the virus category, as at present generally accepted.

It is with considerable pleasure that we acknowledge the help and co-operation so kindly afforded us by many colleagues in Scotland and elsewhere.

Thus it was originally at the instigation of Professor Mackie, the head of our department, that the preparation of this book was undertaken, and we desire to express our warmest appreciation of his many welcome suggestions, constructive criticism, and valued counsel regarding the selection of subject matter, also for the Introduction which he has himself contributed.

To our friend Professor W. J. Tulloch of Dundee we owe our thanks for his many valuable suggestions in connection with the sections dealing with filtration
diseases of
fforded us
smallpox.

[1] Oxford University Press, 2nd edition, 1934

Similarly, Dr. Mervyn Gordon and Lt.-Col. W. D. H. Stevenson of London have extended to us the benefit of their wide experience of vaccination, smallpox, and related matters.

Next we should like to record the willing assistance received from the following members of the Edinburgh Medical School regarding certain subjects in which they are personally interested: Dr. A. E. Cameron (entomological aspects of yellow, sandfly, and dengue fevers); Dr. A. C. P. Campbell (poliomyelitis), Dr. S. W. Challinor (*the preparation of collodion membranes*), Lt.-Col. E. D. W. Greig (lymphogranuloma inguinale, Rift Valley, dengue, yellow, and sandfly fevers), Lt.-Col. W. F. Harvey (inclusions, trachoma), Lt.-Col. J. du P. Langrishe (certain sections on epidemiology); Lt.-Col. W. Glen Liston (yellow fever), Lt.-Col. A. G. McKendrick (rabies, pseudorabies), Dr. W. G. Millar (the microscope), and Dr. A. C. Stephen, Royal Scottish Museum (section on monkeys in yellow fever chapter).

We are very grateful to the following, who have kindly supplied certain photographs to illustrate the text. Col. L. T. Poole of the Royal Army Medical College, London (Negri bodies), Dr. V. Grysez of Lille (preparation of rabies vaccine), Professor E. G. Nauck of Hamburg (inclusions of lymphogranuloma), and Lt.-Col. W. D. H. Stevenson of London (vaccinial infection of the chorio-allantois). Illustrations reproduced from different sources have been individually acknowledged in the text and the remainder have been executed by ourselves, from our own preparations.

We cannot adequately express our appreciation to our colleagues Mr. James A. Ross and Dr. A. T. Wallace who have read the major part of the typescript, and Dr. R. K. Oag who has read all the proofs.

The heaviest burden of preparing this work for press has fallen on our secretary, Miss Frances L. Henderson, M.A., and we are particularly grateful to her *for the skill, untiring care, and painstaking attention which she has devoted to the* task entailed.

The writing of this book has entailed very extensive reference to libraries, and it gives us the greatest pleasure to take this opportunity of acknowledging the invaluable assistance so cheerfully given to us by the following librarians: Miss Charlton and Miss McIntosh of Edinburgh University, Mr. Graham and Miss Russell of the Royal College of Physicians, Edinburgh, Mr. Shields of the British Medical Association, London, *and the staffs of many other libraries throughout* Great Britain and the Continent, without whose patient co-operation the work could not have been accomplished.

In conclusion, we should like to remind the reader that the subject-matter of this book has been compiled, and the proofs corrected, largely during the troubled times between the months of September 1938 and December 1939, and although we have made every effort to avoid incorporating errors in the text, some doubtless will remain, and we therefore beg the indulgence of our readers for those which have escaped detection.

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March, 1940

AUTHORS' PREFACE TO THE SECOND EDITION

The purpose of this book is to provide a volume of reference for both the laboratory worker and clinician interested in the field of human and animal virus

thalmology, respiratory diseases, and veterinary medicine. Likewise it should prove helpful to postgraduates preparing for examinations in these and related subjects.

We have adhered to our original plan of describing only those human diseases of virus origin in which the causal agent has been definitely established. Rickettsial infections of man, virus diseases of animals not known to be transmissible to man, plants, and insects, together with viruses responsible for tumor formation, have not been included.

Nearly ten years have passed since the first edition appeared, and in this time so many advances have taken place in the fundamental and applied aspects of virus research that a thorough revision of the text has become necessary. Thus the following chapters have been rewritten and expanded: The Electron Microscope, Use of the Fertile Egg for Virus Cultivation, Mumps; Yellow Fever, Influenza, Psittacosis, Epidemiology, Diagnosis, and Prevention of Smallpox, Antirabies Treatment; Poliomyelitis; St. Louis, Japanese, and North American Equine Encephalomyelitis. Among virus diseases described in this volume for the first time are, German measles, West Nile disease and related conditions, Colorado tick fever, Newcastle disease, epidemic keratoconjunctivitis, pneumonitis and atypical pneumonia, Venezuelan equine encephalomyelitis, Russian spring-summer encephalitis, infective hepatitis and serum jaundice Altogether some twenty new virus infections have been added, and reference is made to some others of lesser concern.

Today it is probable that virus diseases as a group occupy a more important position in human and veterinary medicine than do those of bacterial origin This fact was demonstrated during the recent war when infective hepatitis, serum jaundice, jaundice associated with yellow fever vaccination, pneumonitis, influenzal diseases, epidemic keratoconjunctivitis, dengue and sandfly fevers, poliomyelitis, and smallpox accounted for much disability over wide areas of the world

Virus diseases now occupy such a major position in the ecology of man and animals that detailed instruction to medical and veterinary undergraduates and postgraduates has become essential, and we hope that this publication will satisfy the needs of teachers called upon to impart such education.

We have acknowledgments to make to many of our friends, and colleagues in Toronto, who have willingly advised us in subjects in which they possess special knowledge Dr Frank Airey (diseases of the skin), Professor E. F Burton (electron microscopy), Mr F W Boswell (electron microscopy), Dr. John Crawley (equine encephalomyelitis and statistical methods), Dr W. E. Coutts (lymphogranuloma), Dr A. F Graham (physical chemistry), and Dr Laurella McClelland (influenza) Drs Crawley, Graham, and McClelland have very kindly assisted with proofreading

In this edition we have made reference to the main contribution to virus literature published up to the end of 1947, and have included some work published in the early months of 1948. For

thanks to many librarians in Lo

Mr Barnard and his assistant

Medicine, the Staff of the Bur

Librarian and his assistants at the British Medical Association Headquarters, Lon-

don; Miss Briggs, Librarian at the School of Hygiene, University of Toronto; Miss Jones and Mr. Wade of the Royal Society of Medicine, London. In conclusion, we acknowledge the generous financial aid of the Sir Halley Stewart Trust, London, which has facilitated the task of revision

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July, 1948

CONTENTS

SECTION 1. TECHNIQUE

SECTION 2. DISEASES OF THE SKIN AND MUCOUS MEMBRANES

SECTION 3. INFECTIVE FEVERS OF VIRUS ORIGIN

SECTION 4. ARTHROPOD-SPREAD AND OTHER TROPICAL INFECTIONS

SECTION 8. RABIES AND PSEUDORABIES

SECTION 9. THE POLIOMYELITIS GROUP

SECTION 10. VIRUS MENINGITIS

SECTION 11. DISEASES CHARACTERIZED CHIEFLY BY ENCEPHALITIS, ENCEPHALOMYELITIS, OR MYELITIS

CHAPTER I

THE MICROSCOPICAL EXAMINATION OF ELEMENTARY BODIES

THE USE OF THE ORDINARY MICROSCOPE FOR THE DEMONSTRATION OF ELEMENTARY BODIES

THE detection of elementary bodies has become a matter of considerable importance in the recognition of certain virus diseases, and in recent years these bodies have been demonstrated in, for example, vaccinia, variola, ectromelia, herpes, varicella, psittacosis, fowl-pox, canary-pox, infectious myxomatosis, and fibromatosis of rabbits. The value of the ordinary microscope for this purpose has been somewhat underrated.

The limit of visibility is stated by Coles (1929) to be 0·074 μ with ordinary white light and 0·0673 μ with green light. The macula of the retina is most sensitive to green light and, owing to the additional contrast which is imparted, a smaller object becomes more easily visible with green than with white light. It must be borne in mind that the employment of mordants for staining elementary bodies increases their size, so that although vaccinia virus may actually be 0·15 μ in diameter by ultrafiltration measurements, after staining it may appear to be 0·3 μ. With improvements in technique it is possible that viruses even smaller than 0·067 μ may be seen as stained particles. There are indications that further developments are likely to occur along these lines, and Merling-Eisenberg (1938), by taking advantage of the fact that the limit of visibility is greater in the dark field, has succeeded in photographing the particles of a *B. coli* bacteriophage which only measured 0·025 μ in diameter. A particle of such size lies on the borderline of visibility, and great skill and care are required to differentiate between virus particles and artefacts. The limit of visibility mentioned above must not be confused with the limit of resolution. The latter is generally stated to be 0·25 μ with direct light; this figure represents the smallest space that could be appreciated by the eye if two objects were placed near to each other. For example, if two parallel lines were gradually brought closer together, as soon as they were less than 0·25 μ apart it would be impossible to discern the gap existing between them and this distance would represent the limit of resolution. Thus, the two images would seem to have fused together and appear as a single straight line, according to Johnson (1928), an objective possessing a numerical aperture of 1.4 is able to resolve a space of 0·2 μ or separate 140,000 lines per inch.

When employing oblique illumination, the limit of resolution or separation is smaller still, and two refractile dots, viewed in a dark field, can be separated if they are situated less than 0·2 μ apart, and by means of darkfield illumination phage particles of the order of 25 mμ in size have been photographed by Merling-Eisenberg (1938).

While the limit of resolution with a lens of 1·4 N.A. is about 0·25 μ, the limit of visibility of this glass when used at full aperture is 0·074 μ for a deeply stained particle seen with ordinary white light, and 0·0673 μ for one viewed in green light (Coles, 1929).

this point more clear, for it will be observed that, although a particle of 0.2 μ in diameter can still be distinctly seen with the naked eye, it becomes more difficult to separate two lines placed 0.2 μ apart. That a gap really exists between these two lines there need be no doubt, for the reader has only to place a magnifying reading glass over the diagram when the two lines will become readily visible. Finally, with the help of the magnifying glass it may also be possible to detect the smallest circle of the series, which measures 0 1 μ in diameter, while two lines situated the same distance apart appear as a single image.



FIG. 2.

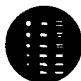


FIG. 3.

FIG 2 Diagram illustrating limits of visibility and resolution The magnified illustration was prepared from an accurate scale drawing made on graph paper in which the largest circle measured 1 cm and the smallest 1 mm The diagram was then reduced ten times so that the largest circle became 1 mm and the smallest 0.1 mm. The drawing has been placed in a circular mask to convey the impress ... whic ... distar ...

FIG ... the view seen under dark ground illumination

By means of these arguments and the assistance of the illustration we have provided, the reader may be able to convince himself that the limit of visibility is smaller than that of resolution

We stated that when employing darkground illumination both the limits of visibility and of resolution were enhanced This can be proved by a study of Fig 3 which consists of a negative prepared from Fig 2, showing the series of circles and parallel lines precisely as they would appear if seen as brightly illuminated objects against a dark background From a comparison of Figs. 2 and 3 it will be observed that the circles and spaces between the parallel lines are more easily visible in Fig 3 than in Fig 2 This is so because a stained particle viewed by direct light is always less easy to see than a brightly illuminated object of the same size placed against a dark background In other words, the contrast between the object and its background is greater in Fig 3 than in Fig 2, which results in increased visibility and particularly resolution when using darkground illumination To some extent, therefore, the limit of resolution can be increased by using a more powerful source of light and increasing the amount of contrast between the object and its surroundings A good example of a practical illustration showing this point is that of the fixed stars seen on a clear night Although these can be seen with the naked eye, they are in reality far below the limit of resolution of even the most powerful telescope, and their apparent magnitude, therefore, depends solely on their brightness against the black sky, thus the brighter the star, the larger it appears to be, irrespective of its real size

Optical equipment The choice of suitable equipment[1] is as important as the correct use of it, and strict attention should be paid to the maker's instructions

[1] The following apparatus has been found useful (van Rooyen, 1937) Patna microscope (Watson) fitted ...

(2) The most suitable substage condensers ... parachromatic dry condenser of N.A. 1 or, ... N A 1 3 or 1 7 With the latter, however, ... ess (see Bridges, 1936, for details regarding an oil-retaining level for oil immersion substage condensers)

The resolving power of a lens has been theoretically explained by Martin and Johnson (1931). Owing to the wave nature of light, an objective is unable to reproduce a sharply defined outline of a minute brightly illuminated object; instead of which it produces an image that consists of a central bright spot surrounded by a series of concentric diffraction rings. This phenomenon has been called the Airy (1834) disk, the radius of which (b') is deduced according to the formula $b' = \frac{0.61\lambda}{\sin U'}$, when λ = the wave length of light employed and U' = the angle between the marginal rays of the lens and its optical axis Hence, if two point objects be brought closer toward each other, it can be proved by means of an intensity curve drawing that the two images will be separated by a dark zone only at such a point when their centers are situated at a distance which is at least approximately equal to the radius of the Airy disk (see Martin, 1925–6).

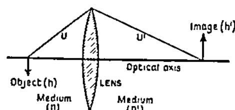


FIG 1. Diagram to explain formation of Airy disk.

By application of the sine relation to an optical system it can be shown from Fig. 1 that $nb \sin U = n'b' \sin U'$, where n and n' represents the refractive indices of the media lying on the object and image sides of the lens and U and U' the angles subtended by the marginal ray and the optical axis. In order to find the smallest distance between two objects conditional with resolution, b' must be substituted by the value of the radius of the Airy disk which is $\frac{0.61\lambda}{\sin U'}$, and accordingly

$$nb \sin U = n' \times \frac{0.61\lambda}{\sin U'} \times \sin U'.$$

But since the refractive index of the medium in which the image is formed is air and this is equal to unity, $b = \frac{0.61\lambda}{n \sin U'}$. But $n \sin U$ is equal to the numerical aperture (N.A.) of the lens (see Mackie and McCartney, 1948, for explanation), therefore $b = \frac{0.61\lambda}{\text{N.A.}}$. It can accordingly be stated that the resolving power of an objective is directly proportional to the wave length of light employed and varies inversely with the numerical aperture of the lens. In the following pages, with the help of a diagram, we shall describe a simple method by which the limit of visibility can be proved to be smaller than the limit of resolution.

The Limits of Visibility and Resolution Diagrammatically Explained

We have just mentioned that when using direct light the limit of visibility is smaller than the limit of resolution. In other words, it is easier for the human eye to perceive a deeply stained particle than to discern a space of the same size lying between two parallel lines. This fact can be demonstrated graphically by means of a scale drawing Let us presume that Fig. 2 represents a microscopic field viewed under ×1,000 magnification, showing a series of circular black dots, the largest of which measures ... se is a pair of parall ... ircle opposite to it ... 1 μ and the last and narrowest is 0.1 μ

A glance at the range of black spots, which may be assumed to represent deeply stained particles in a microscopic field, will immediately show that, whereas a minute black circle of 0.4 μ is readily visible, it is perhaps not so easy to see a gap of 0.4 μ between the corresponding parallel lines The next pair of objects makes

this point more clear, for it will be observed that, although a particle of 0.2 μ in diameter can still be distinctly seen with the naked eye, it becomes more difficult to separate two lines placed 0.2 μ apart. That a gap really exists between these two lines there need be no doubt, for the reader has only to place a magnifying reading glass over the diagram when the two lines will become readily visible. Finally, with the help of the magnifying glass it may also be possible to detect the smallest circle of the series, which measures 0.1 μ in diameter, while two lines situated the same distance apart appear as a single image.



Fig. 2



Fig. 3.

Fig 2. Diagram illustrating limits of visibility and resolution. The minified illustration was prepared from an accurate scale drawing made on graph paper in which the largest circle measured 1 cm. and the smallest 1 mm. The diagram was then reduced ten times so that the largest circle became 1 mm. and the smallest 0.1 mm. The drawing has been placed in a circular mask to convey the impression as seen in a ×1,000 microscopical field in which the largest circle was 1 μ in diameter. The distance between each set of parallel lines corresponds to the size of the adjacent circles.

Fig 3 is a negative print of Fig. 2 and represents the view seen under dark ground illumination.

By means of these arguments and the assistance of the illustration we have provided, the reader may be able to convince himself that the limit of visibility is smaller than that of resolution.

We stated that when employing darkground illumination both the limits of visibility and of resolution were enhanced. This can be proved by a study of Fig. 3 which consists of a negative prepared from Fig 2, showing the series of circles and parallel lines precisely as they would appear if seen as brightly illuminated objects against a dark background. From a comparison of Figs. 2 and 3 it will be observed that the circles and spaces between the parallel lines are more easily visible in Fig 3 than in Fig. 2. This is so because a stained particle viewed by direct light is always less easy to see than a brightly illuminated object of the same size placed against a dark background. In other words, the contrast between the object and its background is greater in Fig. 3 than in Fig. 2, which results in increased visibility and particularly resolution when using darkground illumination. [illegible]

naked eye, they are in reality far below the limit of resolution of even the most powerful telescope, and their apparent magnitude, therefore, depends solely on their brightness against the black sky, thus the brighter the star, the larger it appears to be, irrespective of its real size.

Optical equipment The choice of suitable equipment[1] is as important as the correct use of it, and strict attention should be paid to the maker's instructions

[1] The following apparatus has been found useful (van Rooyen, 1937) Patna microscope (Watson) fitted with rackwork draw-tube, universal mechanical stage *and centering* substage mechanism, compensating eyepieces ×10, ×15, and ×20, apochromatic objectives, 2 mm (1/12 in oil immersion) of N A *1·4 (Leitz)* or 2 mm. holoscopic objective of N A 1·30 (Watson), 8 mm dry apochromatic objective of N A 0·65 (Zeiss), and 6L dry achromatic objective of N.A. 0·65 (Leitz) *The most* suitable substage condensers have been found to be either the (Watson) parachromatic dry condenser of N A 1 or, better the holoscopic oil immersion model of N A *1·3* or *1·7* With the latter, however, the slides must not exceed 1·3 mm in thickness (see Bridges, 1936, for details regarding an oil-retaining level for oil immersion substage condensers).



FIG 4 Photomicrograph taken with the ordinary microscope, showing the elementary bodies of vaccinia viewed in the darkground field. The picture was taken with a Leitz 6L dry high-power objective and ×14 ocular, the total magnification is ×630.

regarding the optimum conditions under which each lens is designed to function. The source of illumination that we have found most suitable for this class of work has been that provided by a short filament high-intensity electric lamp bulb, and a lamp containing a 12-volt 4-amp. bulb should be fitted with a condenser and an

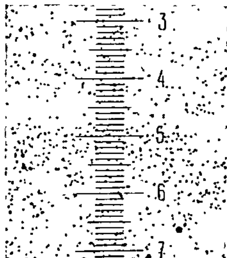


FIG 5. Photomicrograph made from a film preparation of vaccinia virus cultivated in the chorio-allantoic membrane of the chick embryo, showing Paschen or elementary bodies

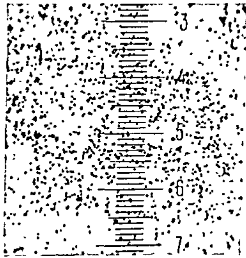


FIG 6 Similar picture made from a film of conjunctival secretion obtained from a rabbit infected with myxoma virus, showing elementary bodies

(Reproduced from *Glas med J*, 1938, *129*, 1)

iris diaphragm for darkfield and critical brightfield illumination, as well as a ground-glass screen for direct observations.

Before examining a preparation for elementary bodies it is necessary to insure that the incandescent filament, substage condenser, objective, and eyepiece are accurately centered and in perfect alignment, for with particles of less than 0.3 μ critical illumination is imperative. Stained films should be made on slides of 1.1 mm. thickness, and should be mounted preferably in cedar-wood oil. It is also advantageous to use only preparations which have been previously compressed between two heavy weights for 24 hours, in order to insure that the coverslip and slide are brought as close as possible to each other. The preparation should be viewed first by direct light with the low-power dry objective to ascertain the types of cells present, and then a 2 mm. oil immersion objective with ×10 and ×15 oculars can be used to search for elementary bodies Fields showing only scanty elementary bodies should be disregarded and, if possible, clumps of them should be sought. Individual bodies or pairs should be moved into the center of the field and carefully scrutinized, first with the ×10 and then the ×15 ocular, the fine adjustment should be continuously used and the height of the substage condenser varied in order to focus accurately the light upon individual particles The substage condenser diaphragm aperture may also be slightly closed in order to give greater depth of focus, and a green filter can be used with added benefit, so as to impart greater contrast to the particle The length of the microscope drawtube should also be adjusted to suit the coverslip and slide thickness (see Martin and Johnson, 1931). Each stained film made from pathological material intended to be examined for elementary bodies should be accompanied, if possible, by two control specimens, one from a similar source of normal tissue, and the other from a specimen which is known to contain elementary bodies, for, unless such controls are included in each series, erroneous conclusions are likely to ensue All three slides should be stained together and examined consecutively. The staining reactions and morphology of any virus bodies having been noted, the same field should next be examined by darkground illumination This can be done by revolving the nosepiece bearing the 2 mm objective away from the slide (which should be firmly fixed to the microscope stage with spring clips), wiping the oil from the surface of the coverslip with a piece of muslin, and then moving either the 6L[1] or 8 mm[1] lens into position The objective should next be focused upon the slide, the ground-glass lamp screen removed, both iris diaphragms fully opened, and a patch stop or a Travis's expanding stop inserted into position in the condenser substage ring Darkground illumination is now obtainable and it should be possible to determine whether or not the stained elementary bodies previously seen in the field are also refractile to obliquely transmitted light (Fig 4) By reversing the steps detailed



FIG 7 Film showing a mixed culture of staphylococci and *B coli* for comparison with elementary bodies shown in Figs. 5 and 6

All three have been stained by the same method, i e Paschen's stain for elementary bodies Each division on the micrometer scale –1.2μ. The magnification –×1,222

(Reproduced from *Glas med. J*, 1938, *129*, 1.)

above, it should be possible to restore the direct illumination for the same field This method enables an observer to study a stained preparation first by direct light and next by darkground illumination, without moving the field or having to do any more than insert a Travis's patch stop into the substage condenser.

Figures 5 and 6 show the appearance of elementary bodies when stained and photographed with the light microscope. Figure 7 may be used for comparison

FLUORESCENCE MICROSCOPY

Hagemann (1937) has demonstrated the value of the fluorescence microscope as an aid to the study of virus bodies, which can be made to fluoresce either by directly irradiating them with ultraviolet light or by first treating them with a fluorescent dye such as yellow 2 G.S, a diazo-amino compound of primulin. When examined under the fluorescence microscope the dyed elementary bodies appear as white or bluish-white particles lying upon a darkly colored background. The method has also been used to examine elementary bodies within living cells.

Sources of ultraviolet light.

Barnard and Welch (1936) state that the best fluorescent effects are exhibited by organisms viewed under darkground illumination and consequently an intense source of ultraviolet light must be used in order to get the best results The carbon or metallic arc may be used, [illegible]
too low an ultraviolet light ou [illegible]

The mercury vapor lamp i [illegible]
is high, its intrinsic brilliancy [illegible]
area, and consequently it is unsuitable for microscopy. Both of the above-described varieties of illuminant are also unsatisfactory because they necessitate the use of a filter in order to absorb the visible rays and there is consequently much loss of brilliancy in the darkground field

[illegible] arge—this is produced between magnesium
[illegible] Wel [illegible]
[illegible] it of [illegible]
[illegible] ioresc [illegible]
ultraviolet light photo-micrography.

Optical equipment The following components are required an ultraviolet light lamp, a quartz or crown glass collecting lens, and a suitable filter. The microscope should be fitted with a quartz darkground substage illuminating condenser and the ordinary glass reflecting mirror replaced by a polished metal one. Fluorescence may be observed visually through ordinary apochromatic objectives with compensating eyepieces, but if photographs with ultraviolet light be required, then quartz objectives must be employed

Material for examination should be spread on quartz slides, treated with primulin (*vide infra*), mounted in Ringer's solution, physiological saline, or distilled water, and covered with a type of cover-glass that is opaque to ultraviolet rays.

Himmelweit (1937) has used vertical illumination[1] for his experiments on fluorescence microscopy and has studied the multiplication of vaccinia virus in the chorio-allantoic membrane of the developing chicken embryo

Procedure.

1 Material containing the virus is spread on a quartz glass slide.
2. It may be fixed, if required, with 96 per cent. alcohol or 1 to 4 per cent formalin for 5 to 10 minutes before staining.
3 Treat with a 0.1 per cent solution of primulin containing 0 2 per cent phenol in distilled water for 15 seconds.

[1] Leitz Ultropak outfit

4 Wash with distilled water, dry, and examine, suitably mounted, under the fluorescence microscope.

When using living tissues phenol should be omitted from the "fluoro-chrome" solution. Primulin is the most effective dye for this class of work and it dissolves in water to produce a bluish fluorescence, which is transferred to organisms impregnated with it.

Hagemann (1937) states that the use of the dye makes it possible to detect certain morphological details that would otherwise be unrecognizable in canarypox, vaccinia, and ectromelia viruses. He also recommends the principle for the morphological study of bacteria and trypanosomes. For other work on fluorescence microscopy see Kufferath (1935), Sutro (1936), and Haitinger (1938).

PHASE MICROSCOPY

Since Zernicke (1935) described this method, further research by Bennett *et al.* (1946) has revealed its possibilities for the examination of unstained biological material The value of the phase contrast technique in the study of virus elementary bodies is yet unexplored.

REFERENCES

See p 20

CHAPTER II

THE MAGNETIC ELECTRON MICROSCOPE

We have already explained on page 2 that the shorter the wave length of light used, the *greater the resultant resolution and useful magnification obtained* More- [illegible] (Hawley, 1945) that to be visible or distin- [illegible] one half of the wave length of light used [illegible] is able to resolve 1/250 inch or 100 μ or 1,000,000 Angstrom Units.[1] Ordinary white light of the visible spectrum has a wave length of *400 to 800 mμ (equivalent to 4,000–8,000 Angstrom Units), a useful* magnification of ×1,200 is attainable with the best optical equipment, the smallest particles discernible in a stained slide are in the region of 67 mμ or 670 A.U.

Ultraviolet light has a wave length of 360 mμ (equivalent to under 3,600 A.U.) and since it is about half that of ordinary light, resolution becomes doubled, a magnification of ×3,200 can be obtained *with increased definition and the morphology* of structures smaller than 67 mμ can be seen. The disadvantage of the instrument is that although a relatively small increase in resolution is gained, the instrument and quartz lenses are hard to fashion, stained preparations cannot be viewed, and the photographic plate is substituted for direct visual observation

The wave lengths of electron rays employed in the electron microscope are extremely short, and measure approximately 0·05 A U. In consequence still higher resolution and useful magnification can be attained up to at least ×100,000 if 10 A.U. are resolved, with excellent definition and great depth of focus Thus, particles of matter possessing very small dimensions, in the region of 10 A U., are brought within the theoretical limit of resolution The limit of resolution of the 1944 Toronto University model is about 50 A U, owing to inhomogeneity of the iron pole pieces By using a special technique with the R C A. model E.M U, resolution of 10 A.U. may be attained with a specimen such as gold particles, this resolution may be limited, however, by lack of sufficient density in biological particles. Readers interested in the theoretical limits of electronic micoscopy should read the publication of Zworykin, Morton, Ramberg, Hillier and Vance (1945).

PHYSICAL PRINCIPLES

The instrument has been developed from the work of Marton (1934, *1934a, 1935, 1936, 1937), Martin, Whelpton, and Parnum (1937)*, von Borries, Ruska, and Ruska (1938), and Burton, Hillier, and Prebus (1939, 1940) The latter effected considerable improvement so that today it is possible to obtain photographs of bacteria and viruses magnified ×100,000 times with good definition The instrument utilizes the physical principle that free electrons and beams of electrons can be deflected by an electromagnetic field To adopt this method for optical purposes, the magnetic fields have been applied in such a way that they act on the electron beam in the same manner as optical lenses do on beams of light Such a field or "magnetic lens" can be produced by a current-carrying coil, as shown in Fig 8 The coil is enclosed in an iron casing which leaves a narrow annular slit at 90° to the axial point where the maximum concentration of field is required. When the current flows through the coil, the free ends of the iron casing are *transformed* into magnetic poles, and between them a magnetic field develops possessing the properties of a convex lens, and the path of a ray of electrons through such a lens is illustrated in Fig. 8 It is, however, complicated by the fact that the electrons,

[1] *Angstrom* Unit = 0·1 mμ = 10^{-} mm = 10^{-8} cm or 1/250,000,000 inch

while passing through the coil, are not only deflected from their original direction in the plane of the drawing, but also spirally around the axis of the coil.

In every single point of the curve, it is only the magnetic component perpendicular to the momentary direction of the ray which acts on it, and produces a

originates which, from
he surface of a spindle.
but can be varied by
the magnetic field and,

consequently, the focal length of the lens. As the focal length varies, the electron-optical image not only becomes more or less sharp, but, over and above that, turns

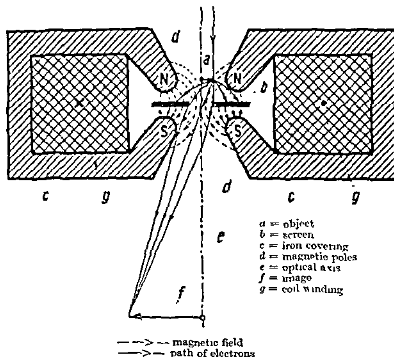


FIG 8 Diagram showing the path of electron rays through an electromagnetic lens, illustrating the principle of the electron microscope constructed by von Borries *et al* (1938).

(Reproduced from *Klin Wschr*, 1938, *17*, 921)

on itself, i e, around the optical axis, in accordance with the spiral ray deflection described Loss of efficiency arising from the latter has been overcome in the latest Radio Corporation of America E.M.U. type model, and resolution in the region of 10 A U has been attained

Since electron beams can exist only in a vacuum, any object which is to be inspected by an electron-optical system must itself be placed within the vacuum, and since electron beams are not directly visible to the eye, the image formed must be made visible either by a fluorescent screen or recorded on a photographic plate

Mechanism of Image Formation

Burton and Kohl (1946) have explained that the principle governing image formation in the electron microscope differs from that of the ordinary light instru-

CHAPTER II

THE MAGNETIC ELECTRON MICROSCOPE

We have already explained on page 2 that the shorter the wave length of light used, the greater the resultant resolution and useful magnification obtained. Moreover, it can be shown diagrammatically (Hawley, 1945) that to be visible or distinguishable an object must be larger than one half of the wave length of light used to illuminate it. The unaided human eye is able to resolve 1/250 inch or 100 μ or 1,000,000 Angstrom Units.[1] Ordinary white light of the visible spectrum has a wave length of 400 to 800 mμ (equivalent to 4,000–8,000 Angstrom Units); a useful magnification of ×1,200 is attainable with the best optical equipment, the smallest particles discernible in a stained slide are in the region of 67 mμ or 670 A.U.

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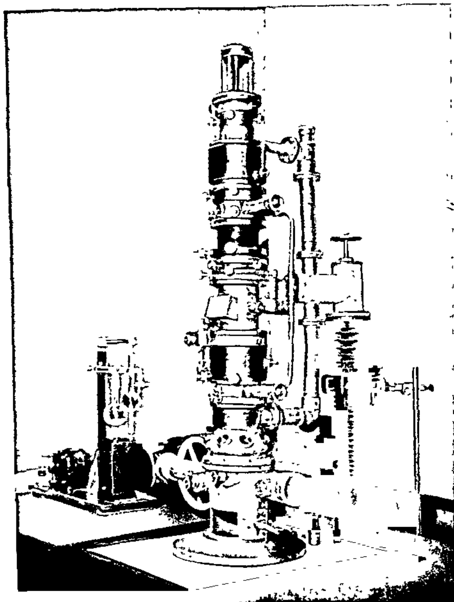


FIG 10 The electron microscope, University of Toronto (1944) type

Industries, Ltd., of Holland, and Metropolitan Vickers, Ltd, of England (Zworykin and Hillier, 1943) Developments continued at the Department of Physics, Toronto University, and Drs Newman and Watson evolved a much improved machine, component parts of which are illustrated in Figs 9–10. Details are as follows Within the electron gun (G) is a tungsten filament, (S) the cathode shield is at the same potential as the filament and produces an electrostatic lens which focuses the electrons leaving the filament The anode (A) is connected directly to the body of the machine and is at earth potential. The condenser lens (C) concentrates the beam of electrons emerging from the central aperture in the

ment. Thus the beam of electrons emitted from the hot filament pass through the electromagnetic condenser lens, and strike the object at uniform speed, whereupon a very few are collected by the specimen, and the bulk is scattered at different angles and varying velocities. Electrons which succeed in passing through the object or near to it without loss of speed are brought to focus on the sensitized photographic emulsion. Dark areas in the final positive print so indicate the existence of matter which has scattered electrons away from the path of the beam

Varying depth of shadows seen in different objects are caused by parallel rays which fall on the object and are scattered to different extents, varying in proportion to the "mass thickness" (i e, density × irradiated thickness) of the object. The greater the mass thickness, the greater is the amount of scattering and the

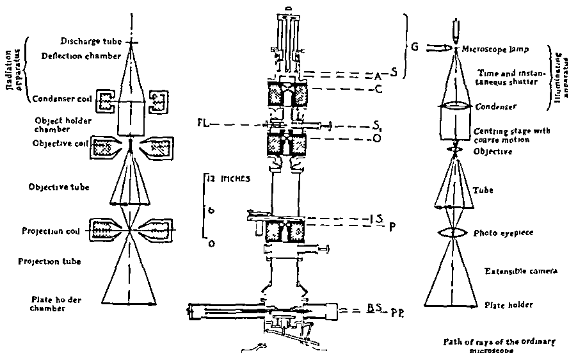


FIG 9 The central diagram illustrates the component parts of the electron microscope built at the University of Toronto in 1944 by Professor Burton and his colleagues (Reproduced from Burton, E F, and Kohl, W. H, *The Electron Microscope*, Reinhold, 1946) The lateral diagrams are reproduced from the earlier description of von Borries *et al* (1938).

(Reproduced from *Klin Wschr*, 1938, *17*, 921.)

darker becomes the picture. The more widely scattered rays are eliminated by not allowing them to pass through the minute aperture of the objective lens. Parts of the object having a greater mass thickness therefore appear darker than those having a lesser mass thickness In this manner, the electron-optical picture can reveal the mass distribution in minute objects

Mechanical, Structural, Vacuum and Electrical Data

The two-stage magnetic lens electron microscope designed by Ruska (1934) served as a prototype for many later instruments. The first to be built on the American continent was constructed by Burton, Hillier, and Prebus (1939, 1940) at the University of Toronto Instruments of similar pattern are today manufactured for sale as commercial articles by the Radio Corporation of America, Philips

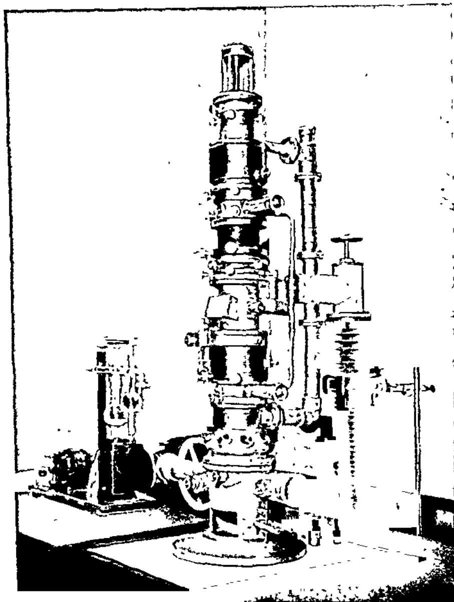


Fig 10 The electron microscope, University of Toronto (1944) type

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anode so as to focus them upon or just above the object specimen. Incorporated inside the condenser lens is a diaphragm with aperture of 0 5 mm that restricts the maximum angle or semi-angle of the cone of electrons descending upon the specimen. (PL) Plunger for lifting and drawing out the object cartridge. (S,) Object air lock seal. (O) Objective lens and (I.S) intermediate fluorescent screen. (P) Projector lens (B S.) Lower fluorescent screen. (P.P) Photographic plate of 2 × 10 inch size, accommodating six exposures of 1½ × 2 inches on each plate.

For optimum results accurate alignment of lenses, object, carriers, and photographic equipment is imperative, and the microscope should be insulated against all external sources of vibration A high vacuum below 10^{-5} mm of mercury is maintained with an oil diffusion pump, in order that the mean free path of the electrons shall be greater than the optical path they have to travel A high vacuum is also essential for the avoidance of gas discharges within the electron gun, and it should be attained as soon as possible after each occasion on which the object or photographic plate is changed.

Burton and Kohl (1946) stress the necessity for a high degree of voltage stability for effective operation of the electron microscope. "Since the focal length of a magnetic lens is a function of the electron velocity, and inversely proportional *to the square of the magnetic field strength, it is very important to maintain the* acceleratı [illegible]
as nearly [illegible]
depends c [illegible]
on the current through their windings.

The range of power and stability requirements of the R.C A. model B microscope are as follows

Circuit	*Range*	*Stability (%)*
Acceleration potential	55 KV	0 002
Condenser lens	20–120 MA	0 02
Objective lens	20–130 MA	0 002
Projector lens	25–125 MA	0 004

HIGH ACCELERATION VOLTAGE (400 KV) ELECTRON MICROSCOPE

It has been shown by le Poole (1947) that by increasing the electron beam energy up to 150 KV, the wave length of the electrons is reduced, which results in theoretical increase in the resolving power of the instrument The use of higher voltages would also appear to enhance contrast in biological specimens. Thus contrast is produced by scattering of electrons in the specimen and the degree of scat*tering is proportional to the mass through which the beam passes.*

In their studies on the passage of electrons through thick objects, van Dorsten, Oosterkamp, and le Poole (1947) draw attention to spatial scattering within specimen Consequently, little relationship exists between the direction of departure of electrons from the object and their point of entry Error due to spatial scattering cannot be corrected by employment of larger objective aperture, but the effect can be reduced by use of higher acceleration voltage, with reduction in average angle of scattering and decrease in chromatic *aberration due to fluctuations in* electron velocity, lesser heating effect in the specimen at the same current density and diminished ionization In an experimental instrument constructed by these workers, examination of dense yeast cells at 350 KV revealed greatly enhanced internal structure and detail which was invisible at voltages of 225 to 100 KV

THE ELECTROSTATIC ELECTRON MICROSCOPE

Bachman and Ramo (1943) have described a compact and simplified variety of this instrument possessing a resolving power of 200 A.U., equivalent to ten times

that of the ordinary light microscope. The electron gun and three lenses are fed from a single voltage supply, and the need for complicated voltage stability apparatus in the power unit is thereby eliminated.

The three electrostatic lenses operate in conjunction, and as long as their voltage ratio is not disturbed, their focal lengths remain the same, irrespective of voltage fluctuations. A magnification of ×1000 may be obtained on the fluorescent screen, and this can be further enlarged without loss of definition up to ×7000. The R C A. Console Model (type EMC-1) electrostatic microscope, giving a magnification of ×5800, has been used by Oster and Stanley (1946) for photographing tobacco mosaic virus

The electrostatic type of instrument has not been extensively used for the study of viruses, and its range of usefulness is still to be explored

PRACTICAL APPLICATIONS OF ELECTRON MICROSCOPY

The electron microscope has made important contributions to knowledge of the particulate structure of crystals, chemicals, colloids, cements, textiles, fibers, dusts, metallic smokes, and a host of related substances. A general understanding of these is indispensable for appreciation of its capabilities in the virus field.

Of considerable benefit is the greatly increased depth of focus which permits having top and bottom of an object simultaneously in focus. Stereoscopic pictures can be made of some materials such for example, as carbon particles, by first photographing the object, then tilting it through a small angle, and re-photographing Thereafter the two pictures are mounted and viewed after the manner of an ordinary stereoscopic illustration. The finer surface structure of polished metals, alloys, crystals, and other opaque materials can be investigated by a different technique. A surface replica representing a mold of the surface of the material is prepared by pouring a solution of formvar[1] over the metal under examination, allowing it to solidify, peeling it off, and then photographing In this way, it is possible to obtain a "negative" of the minute surface indentations of the metal which produce sufficient depths in the thickness of the contact side of the formvar

and Peck (1942). For darkfield illumination in electron microscopy, see Levy (1944).]

General Biology

The surface contours of human tooth enamel have been studied by use of *polystyrene-silica impression films,* in which a polystyrene mold of the tooth is made under pressure The tooth is removed, and a thin layer of silica is evaporated onto the mold The polystyrene is dissolved, and the silica "positive" is examined.
Driest and Muller (1935); diatoms
1941), the coloring of bird feathers
insects by Richards and Anderson
d Ruska (1939), muscle protein by
Ardenne and Weber (1941), tissue cultured chick embryo cells by Porter, Claude, and Fullam (1945), and the structure of collagen and muscle fibers by Hall, Jakus, and Schmitt (1946) The reaction of tobacco mosaic virus and antiserum was studied by Anderson and Stanley (1941) Greases have been investigated by Ellis (1947).

[1] Formvar (Grade No 15-95) is a polyvinyl formol plastic compound sold by Shawinigan Products Corporation, New York.

Histology

Even the thinnest sections cut on the ordinary microtome are too coarse to be penetrated at the usual speed of electrons emitted by the accelerating field of 40–60 KV employed in standard equipment. Increased penetration is possible by use of higher velocity electrons, but unfortunately contrast is lost. Burton and Kohl (1946) state that for 60 KV sections should be less than 0.25 μ thick; sections 0.1 μ thick have been prepared of the striated muscle of the cockroach by Richards, Anderson, and Hance (1942). [For constructional details of a suitable microtome and sectioning method, see O'Brien and McKinley (1943), and von Ardenne (1939).]

Bacteria, Rickettsiae, Viruses, and Bacteriophages

The entire range of virus particles from 10 mμ to 250 mμ lie within the limits of resolution of the electron microscope, and much information regarding morphology has already been obtained by this method (see p 17). The rickettsiae of typhus fever have been photographed by Weiss (1943). In the case of bacteria, the electron microscope has revealed cell walls structurally distinct from inner protoplasm, flagella on motile species, differentiation within the inner protoplasm, and in some cases localized nucleoprotein within the cell, which has been interpreted as a nucleus (Mudd and Anderson, 1944). Selective staining of bacterial cells with heavy metals is also possible (Mudd and Anderson, 1942). More recently, the electron microscope has been used very successfully to follow structural changes during sporulation of *Bacillus mycoides*, and subsequent germination of the endospores, by Knaysi and Baker (1947), and Knaysi, Baker, and Hillier (1947). The latter claim to have demonstrated bacterial nuclei in photographs taken at an accelerating potential of 150 KV.

Mounting of Specimens

A delicate supporting membrane is prepared by immersing a 3×1 inch glass slide in a 0.1 per cent. solution of formvar in ethylene dichloride, this is dried in air, and the film is cut into small square segments with the point of a needle Thereafter, the slide is gently immersed in a bowl of distilled water, the film detached from the glass and allowed to float on the surface of the water. Next, the formvar film is transferred from the water and placed on top of a 200-mesh stainless steel or copper mesh screen, measuring about 3 mm in diameter. This can be done without damaging the film by passing a small metal ladle with a hole in the center beneath the floating film, lifting it from the water, and placing it upon the tiny object screen disk which can be conveniently rested upon an upright and projecting end of cylindrical brass rod.

Films may also be prepared from hot xylene solutions of alkathene plastic, a low polymer of ethylene formed at high pressure, which is resistant to organic solvents (Ellis, 1947 *b*).

The copper mesh screen and membrane covered side can now be inoculated with a drop of infected material, with the aid of a fine bore pipette Much will depend on the concentration of bacterial or virus bodies in the inoculum Thus if the material is dilute, it may be permitted to dry, or even reinoculated if necessary
10^{8} particles per c c, if a drop is hemispherical
centrated, excess bacteria and débris should be
water and the use of absorbent blotting paper,
in such a way as to avoid tearing the surface film. After drying, the preparation should be treated with any fixative such as formalin or picric acid When examining bacteria, it is possible to judge the number of organisms dispersed over the surface of the film by placing the metal screen on a microscopical glass slide and viewing it with a dry high power objective, simultaneously the presence of fissures

and flaws in the film can be detected, and imperfect preparations discarded. If it is decided to photograph the preparation untreated, the screen and film are placed in a cylindrical brass holder, inserted in the appropriate chamber of the electron microscope, the door is closed, the air exhausted with the vacuum pump, fields are searched with the fluorescent screen and multiple photographs taken. A closed cell for electron microscopy has been described by Abrams and McBain (1944).

Electron Micrographs of Shadow-Cast Elementary Bodies

Since the electron microscope permits great depth of focus in working, the thickness of minute objects of molecular dimensions can be measured by observing



Fig 11 Elementary bodies of molluscum contagiosum, photographed without shadowcasting, × 29,000

shadows cast when they are coated with an extremely thin layer of metal which lacks distinctive structure, e g, gold, chromium, or uranium deposited obliquely from a point source in vacuum (Williams and Wyckoff, 1944, 1945). These workers subsequently applied the technique to the study of virus elementary bodies with

brilliant results. A collodion (or formvar) membrane supported on a 200-mesh screen was first inoculated with a pure suspension of elementary bodies obtained as free as possible from tissue, debris, and amorphous particulate matter by differential centrifugalization. Taylor *et al.* (1943) recommend the employment of $CaCl_2$-Ringer fluid as suitable for emulsification of sedimented material, and 0·02 p.c. $CaCl_2$ solution for further dilutions. According to Beard (1945), the low scattering power of viruses can be enhanced by increasing contrast with $CaCl_2$ or $MgCl_2$. Separate collodion films were inoculated with one drop of each concentration of virus, allowed to rest for two to five minutes, and the surplus fluid was

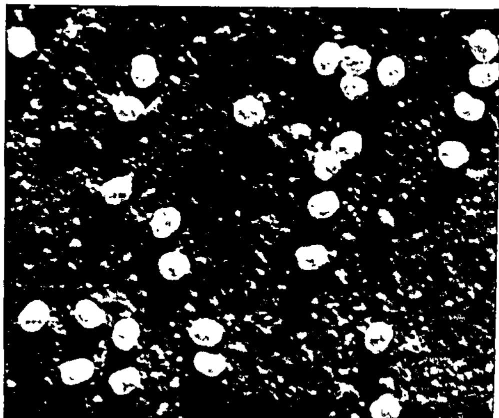


Fig. 12. Elementary bodies of molluscum contagiosum, shadowcast with chromium, × 22,000.

aspirated. After drying, the films and grid object supports were placed in a vacuum chamber, and metal from two hot filaments was evaporated simultaneously at known angles into the specimen. By this means a layer of metal 7 mμ thick can be deposited over the surface of the preparation. The optimum angle at which metallic coating is performed depends on the size of the object, but Williams and Wyckoff (1945) found that for influenza virus (75 mμ) the best angle was that required to make the length of the shadow 5 to 10 times the height of the virus, so that in a successful finished photograph, the elementary bodies should stand out in relief. If accurate height measurements are contemplated, two shadows cast from different directions can be used (see Figs. 11, 12, 13, and 14). Inclusion bodies can also be demonstrated by shadow casting (see Fig. 15 and Boswell, 1947).

Morphology of Viruses

A great number of viruses affecting man, animals, bacteria, and plants have been studied with the electron microscope. Simultaneously, there have been constant improvements in the technique of electron microscopy, the design of instruments and the interpretation and significance of photographic images It should be remembered that when an object is examined with the electron microscope, it is exposed to the effect of a high vacuum as well as slight heating from electronic bombardment, and so a certain amount of shrinkage occurs. It is also possible that other changes may occur in biological matter subjected to prolonged exposure to the

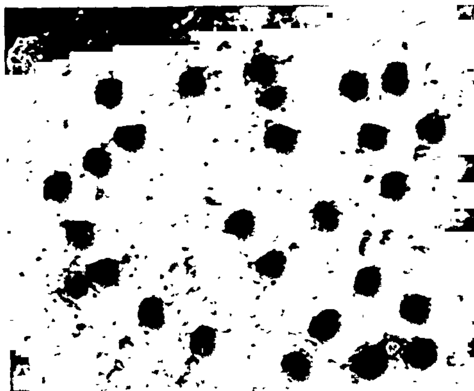


FIG 13. Fowl-pox elementary bodies, shadowcast with chromium, ×20,000.

electron beam Thus, in interpreting the significance of results, a cautious attitude should be adopted, until such time as these expensive instruments are more extensively employed, the results of earlier workers are confirmed, and some degree of standardization is adopted in the selection, manipulation, and mounting of biological material Vaccinia elementary bodies have been photographed, measured, and found to appear as rectangular "brick-shaped" structures occurring in short chains or diploid formation (Green, Anderson, and Smadel, 1942). Likewise, the elementary bodies of molluscum contagiosum, ectromelia, infectious myxoma of the rabbit, and canary-pox present a polyhedral and slightly barrel-shaped outline Ruska and Kausche (1943), and Green, Anderson, and Smadel (1942) found that influenza virus A (PR8 strain) showed a circular outline.

Williams and Wyckoff (1945) estimated that influenza virus A measured 75 mμ, and Mudd and Anderson (1944) reported it to be 100 mμ. Taylor (1944)

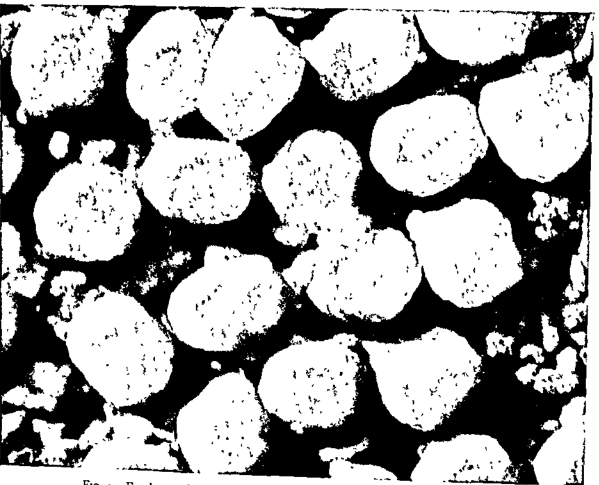


Fig 14 Fowl-pox elementary bodies, shadowcast with chromium, ×80,000.

stated that influenza virus A (PR8 strain) measured 101 mμ, influenza virus B (Lee strain) 123 mμ, and swine influenza virus 96.5 mμ; all were circular in outline Influenza virus B (Lee strain) was said to measure 77.6 mμ by Sharp *et al.* (1944).

Shope papilloma virus was found to be circular in shape and have a mean diameter of 44 0 mμ by Neurath *et al.* (1941). Eastern equine encephalomyelitis virus is spherical in outline and measures 40 2 mμ, according to Taylor, Sharp, Beard, and Beard (1942). The Western variety was photographed at x52,500 diameters and found to be 40 mμ, according to Beard (1945) The virus of New-

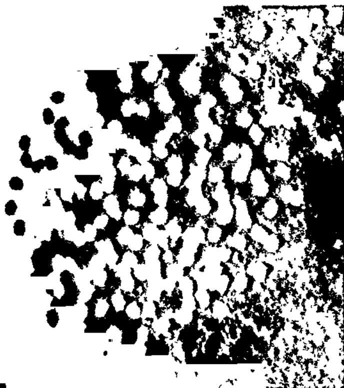


FIG 15. Ectromelia inclusion body, chromium shadowcast, × 12,500

(Figs. 11 to 15 are reproduced by courtesy of Mr. F. W Boswell, M A)

castle disease of poultry has been stated to possess a filamentous phase (Bang, 1947) Normal chick embryo component has been stated to measure 20 mμ (Taylor, Sharp, Beard, and Beard, 1942*a*)

Bacteriophages

Ruska (1941) noticed that when magnified x36 to 41,000, certain types of phage appeared sperm-shaped. Luria, Delbruck, and Anderson (1943) investigated two phages, alpha and gamma, active against *Escherichia coli* Strain B, described earlier by Delbruck and Luria (1942) Virus alpha was found to possess a round head 45–50 mμ in diameter, with a straight or slightly curved tail 150 mμ long and 10–15 mμ thick. Virus gamma showed an oval head 65–80 mμ, a straight tail 120 mμ long and 2 0 mμ thick attached at one pole. It was further noted that each variety multiplied independently on the common bacterial host. Adsorption of

virus alpha on *B. coli* strain B at 37° C. was followed by a pause of 13–17 minutes and lysis of the cell with liberation of about 140 infective units. Gamma virus acting on the same bacterium after an interval of 21–25 minutes, released 135 units per cell. A different coli phage was found to be 50–60 mμ in diameter, without an obvious tail-like appendage. A staphylococcus virus examined was shown to possess a head of 100 mμ in diameter, with a tail 200 mμ in length. The phage particles described were present in amounts proportional to the activity of the material as assayed by plaque counts Furthermore, the structure of the visible particles was found constant for each strain and bacterium exposed. The action of each phage liberated particles morphologically distinctive of the attacking phage.

Luria, Delbrück, and Anderson (1943) also stated that since two unrelated phages were able to parasitize the same bacterial host, it was unlikely that the

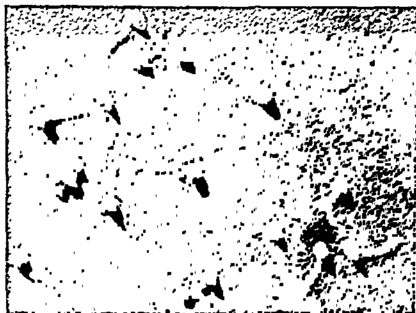


Fig 16 *B megatherium* bacteriophage, shadowcast with chromium, × 40,000
(Reproduced by courtesy of Mr T. A. McLaughlan, M A)

particles freed from the disrupted cell could have been natural protoplasmic components of the bacterium Observations on the same *E coli* gamma (T2) phage, by Hook *et al* (1946), revealed that in crude lysates, the phage resembled a tadpole-shaped particle, with a large head measuring 100×80 mμ, and a short tail 111 mμ by 18 mμ, with knob or disk-shaped extremity An illustration of a B *megatherium* bacteriophage appears in Fig 16 (see McLaughlan, Clark, and Boswell, 1947).

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CHAPTER III

THE STAINING OF ELEMENTARY BODIES

Glass Slides

THE VARIETY found most suitable for preparing films of elementary bodies are those made of crystal glass varying from 1 to 1.1 mm in thickness (Coles, 1935). This size suits the working distance of most microscope substage condensers, and consequently slides less than 1 mm or more than 1.3 mm in thickness must not be used unless a specially designed condenser is employed.

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hours, wash with running water for 24 hours, and store in 50 per cent. alcohol until required, after which they should be wiped and dried with clean gauze. (2) Slides may also be cleaned by boiling them for 15 to 30 minutes in a solution consisting of concentrated sulphuric acid 6 c c, potassium bichromate 6 gm, and water 100 c.c, the liquid being periodically agitated with a glass rod to prevent adhesion of the slides. After removal from the bichromate solution, they should be thoroughly washed in running water for several hours, and then stored in 50 per cent. spirit in an air-tight container.

METHODS FOR THE STAINING OF ELEMENTARY BODIES

The elementary bodies of vaccinia were first demonstrated in stained film preparations made from vesicle fluid by Dr John Buist working at the University of Edinburgh in the year 1886. Buist's observations are of great historical interest, for although he did not recognize the etiological significance of the minute bodies in vesicle fluid, he was undoubtedly the first to have noticed them (Gordon, 1937; Mackie and van Rooyen, 1937).

In a later publication Buist (1887) clearly revealed from an illustration that he not only differentiated these small objects from cocci, but also appreciated the difficulty [illegible] iterest, we ourselves [illegible] by Buist over fifty [illegible] favorably with modern methods

The following is a list of different staining methods which have been found suitable for the demonstration of elementary bodies

Buist's (1887) Method

This is mainly of historical interest, and is carried out as follows

Solution required

Ehrlich-Koch solution consisting of saturated alcoholic solution of methyl violet, 5 to 20 c c, and 100 c c of aniline water consisting of 2 per cent. aniline shaken up with 98 c c of water and filtered

Procedure

1. Cover-glass preparations are made directly from typical vaccine vesicles, and fixed by passing them three times through the flame of a Bunsen burner.
2. Stain for 12 hours in freshly prepared aniline methyl violet solution.
3. If quicker staining is required, then the solution must be gently heated for half to one hour

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It is essential that glass slides should be thoroughly cleaned before attempting to make films of material to be stained for elementary bodies, and the following two methods have been recommended. (1) Place the slides in chromic acid for 24 hours, wash with running water for 24 hours, and store in 50 per cent. alcohol until required, after which they should be wiped and dried with clean gauze. (2) Slides may also be cleaned by boiling them for 15 to 30 minutes in a solution consisting of concentrated sulphuric acid 6 c.c., potassium bichromate 6 gm, and water 100 c c., the liquid being periodically agitated with a glass rod to prevent adhesion of the slides. After removal from the bichromate solution, they should be thoroughly washed in running water for several hours, and then stored in 50 per cent. spirit in an air-tight container.

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Procedure

1 Cover-glass preparations are made directly from typical vaccine vesicles, and fixed by passing them three times through the flame of a Bunsen burner.
2. Stain for 12 hours in freshly prepared aniline methyl violet solution.
3 If quicker staining is required, then the solution must be gently heated for half to one hour

4. Wash in distilled water.
5. Next treat in 60 per cent. alcohol.
6. Dehydrate in absolute alcohol.
7. Mount immediately in balsam and benzol.
8. Contrast staining is usually unnecessary, but if desired, a solution of vesuvin (Bismarck brown) or chrysoidin may be used after washing in 60 per cent alcohol

Paschen's (1906, 1913) Method (see also van Rooyen, 1937)

This method was employed by Paschen for staining the elementary bodies of vaccinia, variola, herpes, varicella, ectromelia, and Shope's fibroma virus, and we have found it to be among the best and most reliable methods for the staining of elementary bodies

Solutions required.

Saturated alcoholic solution of basic fuchsin, 10 c.c., 5 per cent. solution of carbolic acid, 90 c c Alternatively the stain could be made up in two parts: Solution (A) consisting of basic fuchsin, 0 3 gm, ethyl alcohol, 10 c.c., and Solution (B) containing phenol, 5 gm, and distilled water, 95 c c. Mix 1 part (A) and 9 parts (B) and filter before use.

Loffler's flagella mordant. 20 per cent. aqueous solution of tannic acid, 100 c c; ferrous sulphate (saturated solution), 50 c c; basic fuchsin (saturated alcoholic solution), 10 c.c. Allow to stand for several days, and filter before use, mordant improves with age (see von Prowazek, 1912).

Procedure.

1. Make film on slide.
2. Place in distilled water for 3 minutes
3. Dry in air.
4. Fix in absolute alcohol for 5 minutes and treat with ether.
5. Dry in air.
6. Treat with Loffler's mordant and heat gently until steam rises, and allow it to act for 5–10 minutes
7. Wash thoroughly in water
8. Stain with carbol fuchsin solution, gently heating the stain until steam rises for 1 minute, and allow it to remain for a further 10 minutes.
9. Wash rapidly in distilled water and dry with blotting-paper.
10. Mount in neutral mountant or cedar-wood oil

Elementary bodies appear deep red in color when stained by Paschen's method.

The Method of Staining Viruses by Giemsa's (1910) Dilute Solution

This stain has been used by many pioneer workers in their researches on virus diseases Almost every type of elementary body can be stained by this technique, and in skilled hands very beautiful tinctorial effects can be obtained. The stain possesses the advantage of staining red and white corpuscles simultaneously in blood films, but unfortunately it suffers from the drawback of being liable to deposit too readily when standing over a period of time, and moreover different samples of the dye may vary considerably in their staining affinities for elementary bodies (Craigie, 1933) The pH of the tap water used for diluting the stain may also influence the results, if it is unduly acid or alkaline in reaction

Coles's (1935) Method

This is probably one of the best methods of applying Giemsa's stain and has been successfully employed to demonstrate the elementary bodies of vaccinia, herpes, psittacosis, and other virus diseases

Apparatus needed.

A curved glass plate 10 in. long and 4 in. broad, possessing a concave upper surface of about 18 in. in radius of curvature A flat tin box 12 in long, 6½ in broad, and 1 in. deep, fitted with an airtight lid.

Solutions required.

Stain. Giemsa's powder (Grubler), 0 75 gm, glycerine, 25 c c, and industrial methylated spirit, 75 c.c., are thoroughly mixed and well shaken

Differentiating solution. Orange G, 1 gm, tannic acid, 5 gm.; and water, 100 c c.

Procedure.

1. Make thin films as usual.
2. Fix by placing over the mouth of a wide-necked jar—containing crystals of iodine—until the film turns light brown after about ½ to 1 minute exposure (Plimmer, 1913).
3. Treat with absolute alcohol for 5 minutes.
4. To stain, place the film face downward on the curved plate and run underneath it three drops of the stock Giemsa's solution and three drops of methylated *spirit to which is added three times as much tap water.*
5. Place the curved plate in the airtight metal box and allow the stain to act for 24 hours.
6. Renew the stain at the end of the first day and allow it to act for a further 24 hours.
7. Wash the film in tap water, and differentiate it if necessary by placing a drop of the differentiating solution on one end of the film, and allowing it to act for a few moments before washing off with water.
8. Dry the film with blotting-paper, and mount in neutral mounting medium

Hosokawa's Modification of Giemsa's Staining Method

(see Taniguchi *et al*, 1934 *a*)

The elementary bodies of molluscum contagiosum, smallpox, varicella, herpes, zoster, fowl-pox, and rabbit myxomatosis may all be stained by this technique irrespective of whether they occur intracellularly or extracellularly The stained virus bodies appear reddish-purple or bluish-purple We have noticed that Hosokawa's solution has a tendency to overstain the granules of eosinophil polymorphs, and unless the results are carefully controlled, mistakes are liable to occur

Solutions required

Fixative Pure methyl alcohol, 100 c.c, formalin, 5 c c, and glacial acetic acid, 1 c.c

Eosin solution 1 per cent watery eosin solution (Grubler B A.)

Stain Eosin methylene blue, 4 gm, azure I, 0 8 gm, crystal violet, 0 05 gm, these are dissolved in a mixture of methyl alcohol, 250 c c, and glycerine, 250 c c This stock solution is diluted before use (*vide infra*).

Procedure

1. Films are made on glass slides in the ordinary manner, and allowed to dry in air
2. Treat with fixative for 2 minutes, wash in water
3. Flood with eosin solution and heat over a flame until steam rises for half a minute, then wash in water
4. Hosokawa's stain is next applied by adding 1 c c of stock solution to 50 c.c of distilled water, and applying the diluted mixture to the slide for 30 to 40 minutes at room temperature
5. The stain is washed off with water, the slide is dried in air and examined under the microscope

The Taniguchi *et al.* (1934 b) Modification of Giemsa's Method for Staining Elementary Bodies

This method has proved useful for staining the elementary bodies of variola, vaccinia and varicella viruses as well as certain types of cell inclusion.

Solutions required.

Fixative. Pure methyl alcohol, 100 c.c.; formalin, 5 c.c.; glacial acetic acid, 1 c.c.
Stain. 1 per cent. watery eosin-Giemsa solution (Grubler B A.).

Procedure.

1. Make smears and dry thoroughly in air.
2. Fix for 2 minutes in fixative solution.
3. Wash in water.
4. Flood with eosin-Giemsa staining solution and gently warm over flame for ½ minute if staining vaccinia or variola virus, and for 1 minute if films of chickenpox or herpes virus are being treated
5. Wash with water, dry, and examine. Elementary bodies stain bluish-purple.

Craigie's (1933) Method

Solutions required.

(*a*) A 2 per cent aqueous solution of mercurochrome.

(*b*) The stain employed has the following composition: 2 per cent. aqueous solution mercurochrome, 220 Soluble (H.W. & D.), 1 c.c., 7 per cent. aqueous solution M/5 Na_2HPO_4, $2H_2O$ (or Na_2HPO_4, $12H_2O$), 5 c.c.; 1 per cent aqueous solution methylene azure (azur I) (certification No NAz4), 1 c.c., 1 per cent. aqueous solution methylene blue (U.S.P. med.) (certification No NA5), 25 c.c.; and distilled water, 75 c.c.

The constituents are added in the order stated and the mixture, which should not be filtered, retains its staining qualities unimpaired at room temperature for three months

Procedure.

1. Spread films very thinly as in preparing a blood film when working with preparations derived from the skin, tissue scrapings should be suspended in distilled water before films are made.
2. Wash in two changes of distilled water for 5 minutes, dry
3. Fix in methyl alcohol for 5 to 10 minutes, dry.
4. Rinse in distilled water and shake or blot off excess water. Cover film with 6 drops of 2 per cent aqueous mercurochrome for 5 to 10 minutes Rinse rapidly in tap water and later in distilled water, blot off excess water
5. Cover film with stain (6 drops) for 5 to 10 minutes.
6. Rinse off stain as rapidly as possible and blot and dry immediately or, alternatively, simply blot and dry without rinsing. The only part of the technique which requires care is the final step of removing the stain and drying the film, since water, if left in contact with the stained film, tends to dissolve out the dye from the elementary bodies Hence it is necessary to dry the preparation as quickly as possible Elementary bodies stain bluish-purple

Herzberg's (1934) Victoria Blue Stain (Ordinary Method)

Victoria blue was first shown by von Prowazek (in 1905) to be a suitable dye for the staining of elementary bodies of vaccinia. With it the elementary bodies of vaccinia, varicella, herpes, and canary-pox have been demonstrated in smears prepared from infective tissue. Herzberg (1936) has also been successful in detecting

the individual differences in size exhibited by these viruses when compared with one another under the microscope. When stained by this method the individual elementary bodies appear dark blue in color and the dye shows little affinity for staining the cell cytoplasm

Solution required.

The *stain* is prepared as follows 9 gm of Victoria blue 4 R. (Bayer) are dissolved in 300 c c. of distilled water, heated to 60° C. for 30 minutes, placed in a brown-colored bottle for 14 days, and filtered before use

Procedure.

The procedure employed for staining elementary bodies of different virus diseases varies slightly and may be summarized as follows

Vaccinia. Films are made from infected corneal tissue 3 to 4 days after infection, dried in air for 24 hours, placed in distilled water for 10 minutes, dried at 37° C. for 1 hour, stained with 3 per cent. Victoria blue solution for 5 minutes, rinsed with distilled water, dried, and examined (See also Haagen and Kodama, 1934).

Varicella. Thin films are made from vesicle fluid, dried in air for 24 hours, stained with Victoria blue solution for 20 minutes, washed with distilled water, and examined under the microscope.

Herpes. Impression preparations are made from infected rabbit cornea, dried for 24 hours in air, placed vertically in a jar of distilled water for 10 minutes, dried at 37° C for 1 hour, stained with Victoria blue for 30 minutes, rinsed in water, dried, and examined.

Ectromelia Films are made from the feet of infected mice, dried for 24 hours, stained for 10 to 30 minutes, rinsed in distilled water, dried, and examined.

Canary-pox Films are made from infected tissue, stained for 5 minutes, washed in distilled water for 30 seconds using two changes until clear, dried, and examined.

Herzberg's (1934) Victoria Blue Contrast Staining Method

This has been used for demonstrating the elementary bodies of vaccinia and canary-pox, and is carried out as follows

Solutions required

(*a*) *Nuclear red*[1] 5 gm. of aluminium hydroxide are melted in 100 c.c. of hot distilled water, filtered through paper, 0 1 gm of nuclear red added, dissolved by boiling, again filtered, and 0.5 c.c of a 10 per cent. solution of acetic acid added

(*b*) *Light green*[1] 1 gm of the dye is dissolved in 100 c c of distilled water, and 0 5 c c of 10 per cent acetic acid solution is then added

(*c*) *Victoria blue* A 0 1 per cent solution of Victoria blue 4 R solution is prepared by diluting the 3 per cent stock stain already described, to this is added 0 5 c c of 10 per cent acetic acid

Procedure

1 Films are prepared as usual
2 Place in distilled water for 10 minutes
3 Dry in air for 1 hour
4. Treat with nuclear red solution for 15 minutes.
5. Rinse for 30 seconds in distilled water using two changes.
6 Dry in air for 30 minutes
7 Treat with 1 per cent light green solution for 2 minutes.

[1] Hollborn (Leipzig) standard stains

8. Wash with distilled water for 5 seconds, twice.
9. Treat with 10 per cent. tartaric acid for 3 minutes.
10. Wash twice in distilled water for 5 seconds each time.
11. *Stain with 0.1 per cent. Victoria blue solution for 20 seconds.*
12. Wash twice in distilled water as before.
13. Dry in air and examine under the microscope.

Gutstein's (1937) Methyl Violet Stain

This stain has been used for staining vaccinial and other elementary bodies.

Solutions required.

(*a*) *1 per cent methyl violet in distilled water.*
(*b*) 2 per cent. sodium carbonate.

Procedure.

Clean slides in nitric acid, and rinse with alcohol-ether.

1. Prepare films, dry in air or incubator. If much protein is present rinse first in saline, and then in distilled water.
2. Fix film in methyl alcohol for half an hour or more, and place the slide in a dry Petri dish.
3. Mix equal parts of solutions (*a*) and (*b*) in a test-tube, filter immediately on to slide, cover the dish with a lid, and incubate it at 37° C. for 20 to 30 minutes.
4. Rinse in distilled water.
5. Dry in air, mount in cedar-wood oil or liquid paraffin When seen under the microscope, elementary bodies stain light violet

The above staining procedure can be shortened by fixing the film by gentle flaming, pouring the filtered mixture of (*a*) and (*b*) on the slide, and slowly heating it over a Bunsen burner until steam rises. After 3 to 5 minutes, the slide should be washed with water, dried, mounted, and examined in the usual way.

Gutstein's (1937) Modification of Herzberg's Victoria Blue Stain

Solutions required.

(*a*) Victoria blue 4R, 1 gm, alcohol, 10 c.c., and distilled water, 90 c.c.
(*b*) 0.02 per cent potassium hydroxide in water.

Procedure.

1. Invert the film and place the slide face downward on two supporting pieces of capillary tubing placed in a Petri dish.
2. Mix equal parts of solutions (*a*) and (*b*) together, filter at once, and run under slide.
3. Cover the Petri dish with a lid and allow it to stand overnight at room temperature
4. Remove and rinse the slide with distilled water.
5. Dry and mount in neutral balsam or paraffin In a well-stained preparation the elementary bodies should stain dark blue

Castañeda's Method (see Zinsser and Bayne-Jones, 1934, also Castañeda, 1930)

This stain is excellent for demonstrating rickettsiae in films of infective tissues, and Bedson (1937) has recommended it for staining psittacosis virus bodies in the spleen of experimentally infected mice

Solutions required.

Buffer. Potassium dihydrogen phosphate (KH_2PO_4), 1 gm., is dissolved in distilled water, 100 c.c., and added to sodium phosphate ($Na_2HPO_4{,}12H_2O$), 25 gm., dissolved in distilled water, 900 c.c. The mixture is adjusted to pH 7.5, and 1 c.c. of formalin is added as a preservative.

Stain. Methylene blue, 1 gm., methyl alcohol, 100 c.c.

Counterstain. 0.2 per cent. safranine O[1] in distilled water, 1 part; and 0.1 per cent. acetic acid, 3 parts.

Procedure.

1. Spread very thin films of the material on clean glass slides.
2. Stain for 3 minutes with a mixture consisting of 20 c.c. of buffer solution, 1 c.c. of formalin; and 0.15 c.c. of stain.
3. Pour the stain off the slide without washing.
4. Treat with counterstain for 1 to 4 seconds (never more than 5 seconds).
5. Wash with tap water, dry, and examine. Elementary bodies stain deep blue.

Bedson and Bland's Modification of Castañeda's Stain
(see Bedson, 1937)

This method has been employed for staining psittacosis virus particles.

Solutions required.

Stain. This is a mixture composed of phosphate buffer solution (pH 7.0), 95 c.c., neutral formalin, 5 c.c., and borrel blue, 10 c.c.

Counterstain. A 1 per cent. aqueous solution of safranine is employed.

Procedure.

1. Smears or impression preparations are made on clean slides.
2. Fix with methyl alcohol.
3. Treat for 2 minutes with staining solution.
4. Wash with tap water.
5. Counterstain for a few seconds, and dry with blotting paper.

Lépine's Modification of Castañeda's Method

Solutions required.

Stain. A stock solution of stain is prepared by dissolving azure II (Grubler or Gurr) to make a 1 per cent. solution in 0.5 per cent. phenol in water, this is diluted before use by adding 10 drops of the solution to a second mixture consisting of 10 c.c. of distilled water, 10 drops of neutral formalin, and 5 drops of 1 per cent. potassium carbonate. This mixture is heated before being applied to the film.

Counterstain. This consists of safranine diluted 1 in 2,000 in distilled water.

Procedure.

1. Films are made and fixed after the manner described above.
2. Stain with hot solution for 5 to 10 minutes.
3. Wash in tap water.
4. Counterstain with safranine for 5 to 10 seconds.
5. Wash in tap water and dry with blotting paper.

Bedson (1937) has particularly recommended this method, which stains psittacosis E. B.'s deep blue.

[1] National Aniline and Chemical Co., New York.

Six Methods Described by Taniguchi *et al.* (1932) for Staining the Elementary Bodies of Variola

Method one.

Solutions required.

(*a*) Pure acetone.
(*b*) *Mordant.* Add 1 per cent. cadmium iodide to neutral concentrated liquid formalin; the yellow precipitate which is formed is next redissolved by adding the minimum quantity of concentrated hydrochloric acid.
(*c*) Eosin, 0.2 gm., and alcohol, 10 c.c., are mixed together with 5 c.c. of concentrated formalin to which distilled water is added up to 200 c.c., so as to make an orange-red colored solution free from turbidity.
(*d*) Carbol fuchsin solution

Procedure.

1. Thin films are made and allowed to dry in air for 1 to 7 days before staining.
2. Treat with acetone for 1 minute, wash slowly.
3. Mordant with formalin solution for 1½ to 2 minutes; wash with water.
4. Stain with carbol fuchsin solution for a few seconds, wash with water, dry, and examine.

Method two.

Solutions required

(*a*) Sodium hydroxide, 0.5 gm., sodium carbonate, 0.5 gm., and distilled water, 10 c.c., are mixed together, absolute alcohol is added up to 100 c.c.
(*b*) Pure sulphuric acid, 2 c.c., water, 10 c.c., and absolute alcohol up to 100 c.c. are mixed together.
(*c*) Zinc or cadmium iodide, 5 gm., and iodine, 0.5 gm., are dissolved in absolute alcohol, 100 c.c.
(*d*) 1 per cent. aqueous solution of eosin.
(*e*) Ziehl-Neelsen's carbol fuchsin solution.
(*f*) Acetone.

Procedure.

1. Place in reagent (*a*) for 1 minute, wash in water.
2. Place in reagent (*b*) for 1 minute, do not wash in water
3. Mordant in (*c*) for 1 minute, wash in water
4. Treat with eosin solution (*d*) for 30 seconds, wash in water
5. Stain in (*e*) for a few seconds, wash in water.
6. Differentiate in acetone for a few seconds, wash, dry, and examine

The Paschen bodies of smallpox stain pink by this method.

Method three.

Solutions required

(*a*) Sublimate alcohol solution consisting of absolute alcohol, 30 c.c., mercuric chloride, 4 gm., and distilled water, 60 c.c.
(*b*) This consists of a solution of alcohol to which a trace of iodine has been added.
(*c*) 0.25 per cent. sodium thiosulphate solution
(*d*) 2.5 per cent. iron alum solution
(*e*) Heidenhain's iron hematoxylin solution

Procedure.

1. Place in sublimate-alcohol at 60° C for 3 hours, wash in water for 5 minutes.
2. Treat with iodine alcohol for 5 minutes; wash with water for 5 minutes
3. Treat with sodium thiosulphate for 10 minutes, wash in water for 5 minutes.
4. Stain in Heidenhain's iron hematoxylin solution for 24 hours.
5. Differentiate in iron alum.

The elementary bodies appear grayish-blue in color.

Method four.

Solutions required.

(*a*), (*b*), and (*c*) as in Method three.
(*d*) Either acid fuchsin solution is used consisting of 10 per cent. acid fuchsin containing 5 per cent carbolic acid, or else 2 per cent aqueous fuchsin containing 1 per cent. sulphuric acid is necessary.
(*e*) Alcoholic solution of picric acid

Procedure.

1, 2, and 3 as in Method three.
4. Flood the slide with acid fuchsin and heat until the steam rises, allow to cool and repeat.
5. Differentiate in an alcoholic solution of picric acid.

The elementary bodies stain reddish-violet.

Method five (Gram's stain applied to elementary bodies).

Procedure

1. Fix film in ether-alcohol for 20 minutes, wash with water.
2. S[illegible]
3. D[illegible]

Elen[illegible]

Method six.

Solutions required.

(*a*) Sodium hydroxide, 0 5 gm, and sodium carbonate, 0 5 gm, are added to 10 c.c. of distilled water, the total volume is made up to 100 c.c. by addition of absolute alcohol.
(*b*) Pure sulphuric acid, 2 c c, is mixed with 10 c c. of water, absolute alcohol is added up to 100 c c
(*c*) Zinc or cadmium iodide, 5 gm, and iodine, 0 5 gm, are dissolved in 100 c c. of absolute alcohol
(*d*) Carbol methyl violet solution (Gram).
(*e*) Lugol's iodine (Gram)

Procedure.

1. Thin films are made on clean slides and allowed to dry in air for 24 to 48 hours
2. Treat with solution (*a*) for 1 minute, wash in water.
3. Treat with solution (*b*) for 1 minute, do not wash in water
4. Treat with solution (*c*) for 1 minute, wash with water.
5. Stain film with carbol methyl violet for 4 seconds, heat gently until steam rises, wash with water
6. Treat with Lugol's iodine for 30 seconds, wash in water
7. Differentiate with alcohol or acetone, wash with water, dry, and examine.

The elementary bodies stain blue-violet

Goodpasture's (1925) Method

This stain was originally introduced for Negri bodies in sections of rabies material. Subsequently, Goodpasture (1925) modified the stain by the introduction of potassium permanganate solution as a mordant, and employed it for staining the elementary bodies of molluscum contagiosum.

Solutions required.

Stain 20 per cent. alcohol, 100 c.c.; phenol (concentrated and pure) solution, 1 c.c., aniline oil, 1 c.c.; and basic fuchsin, 0 5 gm. The stain should be made up each time before use.

Mordant. 1 per cent. potassium permanganate solution in distilled water is used.

Counterstain. A saturated solution of methylene blue in alcohol, 30 c c., and a 1: 10,000 dilution of potassium hydrate in distilled water, 100 c c.

Procedure.

1. Films should be fixed in Zenker's fluid as the method is nonapplicable to tissues fixed in Helly's fluid.
2. Mordant for 1 minute with potassium permanganate solution, wash thoroughly in water.
3. Stain with carbol-aniline-fuchsin solution for 10 minutes.
4. Wash off the excess stain with water, blot with filter paper, and decolorize with 96 per cent. alcohol until the section or film turns light pink in color.
5. Wash with water, and counterstain for 15 to 60 seconds with Loffler's methylene blue solution.
6. Wash with water, dehydrate, and decolorize for a few seconds in absolute alcohol until the excess of blue is removed.
7. Clear in xylol and mount in balsam.

Morosow's (1926) Modification of Fontana's Method for Staining Elementary Bodies

Solutions required.

Fixative. This consists of acetic acid, 1 c.c., 40 per cent. formalin, 2 c c.; and distilled water, 100 c.c.

Mordant. Carbolic acid saturated solution *Karbolsaure* (see Morosow, 1926), tannin, 5 gm; distilled water, 100 c c

Silver stain. 20 c.c of distilled water plus one drop of 25 per cent. ammonia are placed in a test tube, and a 10 per cent. solution of silver nitrate is added, drop by drop with a pipette, until a brown precipitate appears. The addition of silver nitrate is continued until this precipitate redissolves and the solution turns opalescent.

Procedure.

1. Make a thin film.
2. Dry in air.
3. Place in distilled water for 10 to 15 minutes
4. Dry in air.
5. Treat with fixative for 1 minute
6. Wash with distilled water.
7. Treat with mordant and heat gently for 30 to 60 seconds until the steam rises, but do not allow it to boil
8. Wash thoroughly with distilled water for 30 seconds
9. Add about 0 5 c c. of silver nitrate to the slide and again heat gently for 1 to 2 minutes until the film turns light brown in color
10. Wash thoroughly with distilled water and examine for Paschen bodies

In a well-stained preparation elementary bodies should appear dark brown. In our experience this method has given good results, but we have come to the conclusion that caution is necessary as artefacts simulating virus bodies are liable to be found if the preparation has been overstained. Turevich (1935) has employed silver impregnation methods for staining the elementary bodies of vaccinia

Macchiavello's Stain for Rickettsiae (Zinsser, Fitzpatrick, and Wei, 1938)

Solutions required.

(*a*) 0.25 per cent solution of basic fuchsin made up in buffered phosphate solution of pH 7.4 or distilled water adjusted to pH 7.2–7.4 by addition of sodium hydrate or sodium carbonate.
(*b*) 0.5 per cent. citric acid solution.
(*c*) 1 per cent. aqueous solution methylene blue.

Procedure.

Smear preparations are dried in air, treated with filtered solution (*a*) for 4 minutes, rapidly washed off with solution (*b*) and rinsed in tap water. Treat with (*c*) for 10 minutes, wash with water, dry.

The method has proved valuable for demonstration of extra- and intracellular rickettsiae which stain red.

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CHAPTER IV

THE USE OF EARTHENWARE, PORCELAIN, ASBESTOS, AND GLASS FILTERS

ALTHOUGH at the present time collodion membrane filters have superseded others in the study of viruses, so much pioneer work has been done with the earthenware types that we feel a general account of their characteristics not to be out of place.

Types of Filter

The following different types of filter have been used in the study of virus diseases

(*a*) diatomaceous earth or kieselguhr,
(*b*) unglazed porcelain,
(*c*) asbestos,
(*d*) and glass, their individual characters will now be described.

(*a*) Diatomaceous earth filters.

The Berkefeld. This type is made in Germany and is supplied in three grades according to the degree of permeability to water. these are the coarse "Viel," medium "Normal," and fine "Wenig" filters, called the V, N, and W grades, respectively The V grade is the most commonly employed of the three, and should retain *B prodigiosus* at a low pressure, such as 100–200 mm. of mercury, but if the pressure is raised above this limit, then not only *B. prodigiosus* but also *B. influenzae* will pass through (see Dible, 1932). The N type prevents the passage of bacteria, and the W grade retains the larger filtrable viruses but allows the smaller forms to go through.

The physical characters of the Berkefeld candle have been investigated in detail by Kramer (1927) and Mudd (1922) and their findings will be mentioned later.

The Mandler. This American modification of the German Berkefeld filter is manufactured from kieselguhr, asbestos, and plaster of Paris, and is supplied in three sizes which are graduated according to the results of an air-pressure test after immersion in water The "preliminary" size resists a pressure of 2 to 5 lb per square inch, the "regular" 6 to 9 lb., and the "fine" 10 to 16 lb. per square inch. In construction, the Mandler filter is more robust than the Berkefeld variety but has not been extensively used in the study of viruses

The British Berkefeld filter is constructed differently from the above, is very fragile, and is made only in one grade which, according to our own observations, appears to possess about the same degree of porosity as the German Berkefeld V candle. Like the latter, the lower earthenware end of the bougie is cemented to a metal tube, and at this point fractures and leaks are prone to develop if the filter is roughly handled during cleaning

(*b*) Porcelain filters.

The Pasteur-Chamberland type is made in France and has been extensively used in virus research It is constructed of unglazed porcelain consisting of a composition of kaolin and sand, and since the entire candle is made in a single piece free from joints, it possesses a mechanical advantage over the Berkefeld type. The Pasteur-Chamberland filter also possesses the added virtue of being easily cleaned by heating the candle to a high temperature in an electric furnace

Nine different grades of these filters are available, namely the L_1, L_1 *bis*, L_2, L_3, L_5, L_7, L_9, L_{11}, and L_{13} sizes The L_1 is the coarsest filter of the range and the

makers state that it is only intended for clarifying purposes, whereas the L_1 *bis* and L_2 candles retain the larger bacteria but allow the smaller micro-organisms such as, for example, the organism of bovine pleuropneumonia to pass through An excellent summary of the general characters of the Pasteur-Chamberland range of filters is given by Dible (1932).

Galloway and Elford (1931), working with foot-and-mouth disease virus, adversely criticized the value of the Pasteur-Chamberland filters and stated that they found them to be extremely unreliable.

(*c*) **Asbestos filters.**

The Seitz. Several sizes are made, the smallest of which is the most convenient for virus work, and consists of an asbestos disk clamped (at its circumference) between two metal surfaces After each experiment, the disk is discarded and replaced by a fresh one (which can be sterilized by autoclaving). Two grades are supplied, namely the K (coarse) grade, intended for clarification, and the E K (fine) for retaining large bacteria and even small organisms such as *B. prodigiosus* or *Br. melitensis*, provided that the pressure is not raised above 100 mm of mercury. We have used the E K grade and found that the viruses of vaccinia, ectromelia, Shope's rabbit fibroma, infectious myxomatosis of rabbits, and bacteriophage readily pass through The permeability of the Seitz filter can be decreased by inserting two disks into the metal mantle, this should be done if at any time difficulty is experienced in withholding bacteria

(*d*) **Glass filters.**

The frittered glass variety is constructed entirely of glass and possesses certain advantages, but such filters have not been widely used for virus work owing to the ease with which they become clogged. A [illegible] able and the coarsest grade, which is only [illegible] large tissue particles before attempting tc [illegible] perhaps the most useful of the whole range. The efficacy of the filter for clarifying may be improved by spreading a layer of asbestos pulp (made by shredding a Seitz disk and soaking it in saline) over the glass filter-bed surface before adding the material

The finer grades of frittered glass filters are made in a great range of pore sizes and any dimension varying from 250 μ to 0 15 μ can be constructed to order. The manufacturers[1] supply data with each filter, stating the diameter of the largest pore, the average pore, and the filtration speed in c c. of water per minute We have tried one model possessing an average pore diameter of 0 667 μ, the largest pore being 0 91 μ, and with a filtration speed of 50 c c of water in 30 minutes, 1 second, for studies on myxoma virus and found it satisfactory The only disadvantage we have experienced with these filters is that the glass pores soon become clogged unless the material is well diluted before attempting to filter.

The Cleaning of Filter Candles

[illegible] in [illegible] tair [illegible] 2 per cent phenol solution

The Berkefeld type should be cleaned, immediately after use, by forcing a current of water in the reverse direction through the filter pores After washing away most of the large particles which clog the pores of the candle, it should be immersed in a bowl of water containing a 2 per cent solution of "liquor trypsini co" (B P) and left for 24 hours, preferably in an incubator at 37° C The candle should

[1] Schott & Gen, Jena, Germany, and Pyrex

next be thoroughly washed, scrubbed with a brush, tested for patency, dried, wrapped up in Kraft brown paper, and resterilized by boiling.

The Pasteur-Chamberland variety can be cleaned in the same manner, or by placing the candles in a cold electric furnace and gradually heating them to white heat. After half an hour's heating the current is switched off and the candles allowed to cool slowly.

Frittered glass type Before using a new filter, it should be cleaned by sucking water through its pores followed by hot hydrochloric acid and water again, to remove organic matter. The filters are sterilized by first moistening them and then placing them in a cold Koch steamer which should be gradually heated, and after one hour's exposure at 100° C. the steamer should be allowed to cool before removing the filter. After use, the filters can be cleaned by immersing them in hot sulphuric acid to which has been added a mixture of sodium nitrate and sodium perchlorate for a period of 24 hours. Sulphuric acid and sodium bichromate are not recommended for cleaning as they tend to affect the electrical charge of the glass pores (Laug, 1934).

Some Theoretical Considerations Concerning the Process of Filtration through Earthenware Candles

A variety of different factors determines the ability or inability of a particle of fixed size to traverse the pores of an earthenware filter, for example

1 The amount of positive or negative pressure applied.
2 The length of time for which it is exerted
3 The viscosity of the material filtered
4. The pH of the fluid in which the tissue is suspended.
5. The number of infective elementary bodies in the suspension
6. The average diameter of the elementary bodies or other infective particles present
7. The electrical charge of the filter and of the material to be filtered
8. The average pore diameter of the filter.

In view of the many factors involved, consideration must be paid to each of these before any virus can be definitely asserted to be filtrable. Special care must also be taken to ensure that the filter candle is free from structural defects such as cracks or flaws.

The Pasteur-Chamberland jointless type of bougie filter is probably the most satisfactory of the earthenware varieties, but it unfortunately suffers from certain serious disadvantages For example, when filtering feebly infective emulsions through such a candle, much of the infective material (elementary bodies) becomes adsorbed and retained in the pores of the candle Thus the filtrate may be inactive, even though the average pore diameter of the filter may be many times greater than the size of the virus These remarks are applicable to the Berkefeld candle and, perhaps to a lesser extent, the Seitz asbestos disk

The adsorptive action of earthenware filter candles on viruses can be demonstrated by the following experiment Prepare a series of five decimal dilutions of desiccated rabbit's testis, infected with vaccinia virus, in saline, ranging from 1 1,000 to 1 10,000,000, and inoculate 0 2 c c of each dilution into the shaved skin of a rabbit Make up about 25 c c of a 1 100 dilution of the same tissue, pass it through a Berkefeld V filter, prepare a second series of decimal dilutions of the filtrate, ranging from 1 1,000 to 1 10,000,000, and inoculate 0 2 c c of each as previously into the skin of the rabbit, alongside the first series Examine the animal daily and note the sequence in which vaccinial papules develop, and by comparing the skin reactions produced by each set of dilutions the loss of infectivity of the filtrate due to adsorption will become evident Much will depend on the individual factors prevailing at the time of performing the experiment, but on one occasion

we observed that, whereas 0·2 c.c. of a 1:1,000,000 dilution of (unfiltered) virus emulsion produced a reaction, the same suspension after filtration only reacted up to 1:100 dilution. In another experiment on Shope's rabbit fibroma virus, the filtrate proved to be completely inactive, although the elementary bodies are no larger than those of vaccinia virus. On the other hand, we have observed that ectromelia virus, which is the same size as vaccinia virus (Elford, 1933), traverses the Berkefeld filter with far greater ease. The question thus arises as to what are these differences attributable, and in discussing this problem, Galloway and Elford (1931) have suggested that the activity of a filtrate is governed by the relative number of elementary bodies present in the tissue emulsion before filtration. Thus the greater the number of elementary bodies in the suspension, the more infective the filtrate will be.

It is also probable that a second factor may be the virulence or pathogenicity of the individual virus particles for the species of animal inoculated For example, the British domestic rabbit is highly susceptible to infection with rabbit myxoma virus, less susceptible to vaccinial infection, and comparatively resistant to Shope's fibroma virus, so that if a rabbit were to be inoculated with a filtrate of one of these agents then the result would depend on the number of elementary bodies in the filtrate To quote a hypothetical instance, the minimum infective dose of Shope's fibroma virus may be 50 elementary bodies, that of vaccinia 10, whereas only 1 elementary body of myxoma virus may suffice to infect a rabbit. The inactive filtrates obtained on passing Shope's fibroma virus through an earthenware candle may, therefore, have been due partly to loss of elementary bodies during filtration, and partly to the natural resistance of the British domestic rabbit to infection with this virus Before it is possible, then, to assess the results and relative significance of filtration experiments with earthenware filters, it is necessary to consider the susceptibility of the test animal to infection, in addition to taking into account the other factors enumerated

The Berkefeld filter is made of diatomaceous earth or a compound of silicic acid and carries an electronegative charge. Mudd (1922) has shown that the pores of the Berkefeld candle are the site of an electrical potential difference consisting of a Helmholtz double layer, in which the wall of the filter is negatively charged and the liquid within positively charged When suspensions of tissue are passed through the pores of such a candle, positively charged particles contained in the material become adsorbed and retained by the filter. Consequently all filters constructed of siliceous material, sand, porcelain, powdered glass, colloidal silica, or diatomaceous earth will adsorb basic dyes but allow acid dyes to pass through Thus Victoria blue, which is a basic dye, will not pass the Berkefeld candle, but [illegible]

will not do so Should, however, the Congo red be diluted and made acid (following which it turns blue), then it will pass the plaster of Paris filter but not the Berkefeld variety Thus by reversing the electrical charge, Kramer succeeded in reversing the filtrability of a dye It was also found that if a filter was constructed of both plaster of Paris and diatomaceous earth, it acquired a neutral charge and permitted both positively and negatively charged dyes to pass its walls The behavior of dyes when subjected to filtration through earthenware candles is similar to that exhibited by positively or negatively charged colloids and viruses, and thus Kramer (1927) found that a *Staphylococcus* bacteriophage would pass through a Berkefeld filter but not through one constructed of plaster of Paris, the same applies to vaccinia, rabies, and tobacco mosaic viruses which are negatively charged Likewise, diphtheria toxin behaves in a similar manner, but tetanus toxin, which is electropositively charged, is adsorbed by the negatively charged Berkefeld filter, although each filter adsorbs only a small quantity of toxin Apart from the

influence exerted by the charge of the filter candle over the transit of a small particle through its pores, another factor which plays an important role is the reaction of the fluid in which the virus is suspended. Many interesting observations have been reported on this subject, and it has been shown that substances which are normally retained by a filter can be made to pass through it by preliminary treatment of the candle with oil, egg white, peptone, or heated serum (see Muir and Browning, 1908–9, Holderer, 1912). For example, according to Grinnell (1929), Victoria blue B which normally does not pass the Berkefeld filter can be induced to go through it if the dye is suspended in hormone broth. Results analogous to the behavior of dyes and other substances have been reported in connection with viruses For example, Ward (1929) has shown that the most active filtrates of vaccinia virus were obtained after emulsifying the vaccinial pulp in hormone broth of pH 7.6, and Ward and Tang (1929) demonstrated that vaccinia and herpes "encephalitis" virus were more easily filtrable when emulsified in hormone broth than in normal saline. Bronfenbrenner (1927) discovered that, if particles carrying a bacteriophage were deposited on a Berkefeld filter, the phage could be washed through the candle with broth, but not with water.

Sawyer and Frobisher (1929) reported that yellow fever virus was able to pass through the Berkefeld N candle when suspended in broth, but not if the infected tissues were emulsified in physiological saline. Foot-and-mouth virus has also been shown to have an optimum filtration range lying between pH 6.4 and 8.75 (see Galloway and Elford, 1931).

These findings suggest that suspensions for filtration should be prepared in broth and made alkaline before attempting to filter them through earthenware candles. The opposite opinion to this has been expressed by Green and Eagles (1931), who filtered large quantities of vaccinia virus through Berkefeld V, N, and British Berkefeld filters, and found that the hormone broth was not superior to distilled water as a diluent. They also maintained that the pH of the virus material did not seem to be important within the range pH 6.4 to 8.4. Furthermore, the lining of filters with egg white did not, in their opinion, enhance filtrability of the virus.

Practical Considerations

Owing to the loss of virus resulting from adsorption during filtration through earthenware candles, the material to be filtered should possess an initial high degree of infectivity At the same time, the concentration of tissue protein should be low and any large tissue particles in the suspension, liable to clog the pores of the filter, should be eliminated by preliminary centrifugation at 3,000 r p m. for half an hour, followed by filtration through coarse asbestos pulp similar to that employed by Laidlaw *et al* (1935) in their studies on the influenza virus These workers freed emulsions of lung tissue from excess organic matter by first passing the extracts through sterile asbestos pulp spread over the surface of a coarse-grade frittered glass filter, after which the material was diluted in saline and filtered through a collodion membrane When using Elford's membranes, only a very low pressure, e g, from 20 to 50 cm of mercury, is required for filtration, with earthenware or Seitz filters a higher pressure is demanded, and this may be applied as positive force on the surface of the tissue extract, or by creating a partial vacuum inside the filter flask The latter is more often used and the "negative pressure" applied should be as low as possible, free from fluctuation, and never exceed 30 cm of mercury if possible The time occupied in filtration, the volume filtered, the temperature of the room, and the prevailing atmospheric pressure should be recorded with the other experimental data

Having removed the filtrate, at the conclusion of the experiment, the filter candle should be tested for flaws and structural defects. This is done by making a dilute suspension of *B prodigiosus* (a loopful of a 24-hours' culture of the organism

in 20 c.c. of saline solution) and adding it to the mantle. According to Grinnell (1929), an old culture of *B. prodigiosus* should be employed as a young culture sometimes fails to pass through a filter which is permeable to an older growth. The bacillary suspension is next filtered under the same conditions as the virus material, and after about 20 c.c. have passed through, the filtrate is tested for the presence of bacteria by adding 2 c.c. amounts to 6 tubes containing glucose broth which are incubated at 37° C. for a week, 2 c.c. should also be inoculated into Robertson's bullock heart medium (Mackie and McCartney, 1948) and incubated at 37° C. for 3 weeks for evidence of anaerobic organisms. If growth is absent, the candle may be assumed to be free from defects. It is sometimes the practice to filter the virus suspension and *B. prodigiosus* simultaneously and this procedure can be adopted if desired, when filtering feebly infective material, it is advisable to avoid this method, since Levaditi and Nicolau (1923) have demonstrated that rabies, vaccinia and herpes viruses can be wholly adsorbed by larger bodies such as carbon particles.

The Interpretation of Results

If the filter candle retains *B. prodigiosus* and the filtrate proves pathogenic to a susceptible animal, the result may be regarded as evidence in favor of the filtrable nature of the infective agent. The experiment should, however, be repeated, using another filter of the same type, and if similar results are obtained the virus may be concluded to be filtrable. It is also advantageous to ascertain which filters the virus fails to pass, and accordingly candles of different porosity should be tested.

If the filtrate proves to be nonpathogenic on animal inoculation, the test must be repeated several times before abandoning the experiments, and the pressure should be increased as much as possible, short of allowing the control organism (*B. prodigiosus*) to pass through the candle. The infectivity of the filtrate can be concentrated either by spinning it in a high speed centrifuge at 15,000 r.p.m. for one hour, or by diminishing the bulk of the fluid by evaporation from the frozen state.

Should all these measures fail to yield an infective filtrate, then one of two alternatives is possible: either (*a*) the infective agent is too large or else (*b*) it has been completely adsorbed by the pores of the filter.

In conclusion, it may be stated that when employing earthenware, porcelain, and similar types of filter, a positive result is of value provided that the filter has been proved to be efficient, but in the case of a negative result being obtained, it does not necessarily imply that the agent is not filtrable, since adsorption effects produced by these filters are sometimes so powerful that certain viruses, e.g., trachoma, have the greatest difficulty in traversing them.

The introduction of Elford's collodion membrane filters has helped to overcome many of the difficulties referred to in the previous pages, and in recent years their value in the field of virus research has been accorded universal recognition. The collodion membrane filter occupies a class by itself, and is eminently suitable for the filtration of viruses, because, owing to its thinness, there is little loss of infectivity resulting from adsorption of virus in its pores, and the comparative rapidity with which filtration is accomplished renders clogging of its pores less liable to occur in comparison with earthenware candles. Furthermore, collodion membranes can be accurately graded in porosity according to their average pore diameter, so that the size of the virus can be measured from the filtration data. The properties of these filters will be discussed in the following chapter.

REFERENCES

See p. 47

CHAPTER V

ELFORD'S COLLODION MEMBRANE FILTERS

THE INTRODUCTION of these filters has greatly advanced the state of our knowledge concerning the relative size of different filtrable viruses, since they have, to a large extent, overcome the undesirable adsorptive effects associated with earthenware filters. The basic principle underlying their construction depends on utilization of *the antagonistic action of amyl alcohol* and acetone, which affects the rate of aggregation of nitrocellulose particles during evaporation while the membrane is in process of formation Thus, whereas individually amyl alcohol and acetone are solvents of nitrocellulose, when present together they tend to coagulate the nitrocellulose, and the alcohol and ether present maintain this gelling tendency. Elford's *ultrafilters have been evolved from a series of systematic studies on the physical* properties of collodion films, during which it was discovered that collodion films exhibited one of two different types of structure Either the membrane showed a coarse microgel appearance, consisting of a microscopically visible structure, or alternatively a finer ultragel composition which could only be seen to be particulate matter when viewed under *the high power magnification of an ultraviolet light* microscope.

Acetic acid and collodion membranes tended to show both the microgel and ultragel construction and were consequently unsuitable for filtration experiments in view of their excessive variability in pore size Alcohol-ether membranes, on the contrary, consisted of the fine ultragel formation, and being of greater uniformity in average pore size, proved more suitable as membranes for the filtration of viruses Elford also investigated the conditions governing the formation of ultragel structure and found that the ultragel structure was consequent upon spontaneous gelation of the collodion prior to the washing process in water, which served to fix the already potentially existing structure. From this information, Elford was able to increase the state of aggregation in nitrocellulose, while the system still retained its gelling properties This was accomplished by incorporating nonvolatile coagulants with the solvents in the collodion, so that, as the solvents evaporated and coagulation commenced, the nitrocellulose became more aggregated. Thus, the individual particle size of the units composing the ultragel tended to become larger, and *synchronously the interstices of the membrane became enlarged, thereby* increasing the permeability of the filter. A number of nonsolvents was tested in order to find the most suitable one for increasing the permeability of the collodion membranes, and water was found to be the best, yielding membranes of good tensile strength The greater the quantity of water added, the more permeable *became* the filter, until finally a maximum volume of 5 per cent. of water produced complete coagulation of the nitrocellulose, yielding comparatively large pores that were only capable of arresting *B prodigiosus.* Decrease in the permeability of the membrane was, on the other hand, produced by the addition of small quantities of ethyl alcohol, methyl alcohol (synthetic), or acetic acid Thus, by incorporation of solvents or nonsolvents in the collodion system, a series of membranes was produced possessing a range of average pore diameters (A.P.D) varying from A P.D. 3 μ to A P.D 10 mμ Such values of A P D. were obtained from observation of the rates of water-flow (R F W) through the membranes and determination of the specific water content (S W C) of each particular grade of membrane

(Elford and Ferry, 1935). The measurements of R.F.W. were carried out by placing a section of membrane between the two limbs of a specially designed U-tube apparatus. In earlier experiments (Elford, 1931) the maximum pore size (M.P.S.) was determined from measurement of the critical air-pressure (C.A.P.)

Notes on the Preparation of Membranes

Workers desirous of making their own membranes are advised to consult Elford's original publications on the subject before attempting to do so (Elford, 1929, 1931, 1933; Elford, Grabar, and Ferry, 1935), as well as the work of Asheshov (1933) and Bechhold (1907, 1908, 1908 *a*). The following notes indicate the general principles on which they are prepared.

Apparatus.

The collodion is poured into a glass cell of special dimensions where it is allowed to evaporate. The cell may be either of the 20 or 40 cm. size. Each cell is constructed of two thick squares of polished plate glass that have been cemented together with egg white, and the upper of these has a circular hole, 20 (or 40) cm. in diameter and 1 cm. deep, bored into it. The glass cell should be placed perfectly horizontal, and its position should be verified with a spirit-level gauge. All apparatus and solutions should attain a uniform temperature before use, and should remain for 1 hour at 22.5° C. prior thereto. Points requiring care are that the volume of the collodion solution should be accurately measured (75 c.c. collodion for a 20 cm. diameter cell), gently poured, and the period of evaporation accurately timed (75 minutes). The degree of humidity of the atmosphere should also be controlled.

Reagents.

Nitrocellulose–Necol collodion No. 365A/9[1] has been principally employed.

As solvents ethyl alcohol, methyl alcohol, ether, glacial acetic acid, acetone, and amyl alcohol are required. These should be chemically pure, according to Elford, Grabar, and Ferry (1935), but Bauer and Hughes (1935) state that reagents of analytical grade are satisfactory without the additional purification recommended by Elford (1931).

With regard to solutions, amyl alcohol and acetic acid are added by volume and the remainder should be prepared on a gravimetric basis. They should be thoroughly mixed by placing them in a mechanical shaker for 4 hours at laboratory temperature before being used. Every procedure should be uniform and constant throughout and only small quantities of material made up at a time.

Nomenclature.

Elford has introduced a notation in order to describe the composition of the different collodion systems employed. Thus "N*a*/40 (*x* *y*)" means Necol collodion containing "*a*" c.c. of amyl alcohol per 40 gm. of Necol (stock), diluted by the addition of "*x*/10" of its own weight of alcohol and "*y* 10" of ether. Any further modification of such a system is denoted as a volume addition per 100 gm. Therefore, N*a*/40 (*x* *y*)+1 per cent. acetic acid means that 1 per cent. of the acid has been added per 100 gm. of the denoted system.

The following data indicate the properties of membranes prepared from N*a*/40 (1 9) systems consisting of "*a*" c.c. of amyl alcohol added to 40 gm. of Necol solution, then diluted with its own weight of a mixture of alcohol and ether in the rates of 1 9 parts by weight.

[1] A standard ether-alcohol collodion of Nobel Chemical Finishes, Ltd., Slough.

Conditions.

These are indicated below (Elford, 1931).

TABLE 1

System	R.F.W.	A.P.S.	C.A.P.	Membrane thickness	Remarks
		μ	lb./sq. in.	mm	
N10/40 (1.9)	11.5	0.80	30	0.31	
" + 0.5% H_2O	13.0	0.87	28	0.31	
" + 1.0% "	17.2	1.0	22	0.32	Conditions
" + 2.0% "	78.0	2.15	12	0.32	Vol. collodion 75 c.c.
" + 0.5% HAc	1.2	0.26	175	0.33	Cell diameter 20 cm.
" + 1.0% "	0.17	0.10	>200	0.35	Temperature 22.5° C.
" + 1.5% "	0.065	0.06	>200	0.36	Evaporation period
" + 5% MeOH	0.75	0.20	180	0.34	75 mins
" + 10% "	0.23	0.114	>200	0.40	
" + 20% "	0.13	0.088	>200	0.45	

From the above it will be observed that a series of membranes ranging from A.P.S. (or A.P.D.) 2.15 μ to 0.06 μ are obtainable.

Summary of procedure.

N10/40 (1.9) parent collodion under the conditions stated yields a membrane ranging from an A.P.D. of 0.65 μ to 0.85 μ. To increase permeability, small amounts of water in quantities of 0.25 per cent. are added so that a maximum A.P.D. of 2 μ is reached when 2 per cent. of water has been added.

To decrease the permeability of the membrane, acetic acid is added and the concentration is increased by 0.5 per cent. each time so that when a total of 2 per cent. acetic acid is reached a membrane of A.P.D. 10 mμ results. The intermediate pore sizes are obtained by varying the evaporation period, and in this way a graded series of membranes ranging in A.P.D. from 3 μ to 10 mμ can be prepared.

Washing of membranes.

This is done in sterile distilled water which is changed thrice daily for a period of 14 days. Alternatively, they may be washed for 4 days in distilled water, 2 days in freshly boiled 20 per cent. alcohol, and a further 4 days in distilled water.

Sterilization and storage.

Membranes can be sterilized either by placing them in cold distilled water, followed by boiling for 1 to 3 hours, or, alternatively, by carrying out some of the later processes of preparation in an ultraviolet light chamber, after which they can be stored in sterile distilled water.

Determination of A.P.D.

Before use, the pore size of each new batch of membranes should be measured by ascertaining the rate of water-flow pressure (R.F.W.) and the specific water content (S.W.C.) of each particular grade of membrane. The following quotation from Elford's (1931) publication describes the method employed for measuring the rate of water-flow.

Rate of flow of water (R.F.W.).

"The time taken for a known volume of distilled water to flow through a membrane of known area and thickness is measured under standard conditions of temperature and pressure. From the data then known it is possible to calculate the rate of flow of water through the particular membrane under certain arbitrarily fixed conditions. By definition

$$\text{R.F.W.} = \text{c.c./sq. cm./min. for} \begin{cases} \text{0.1 mm. membrane thickness.} \\ \text{100 cm. water pressure} \\ \text{20° C. temperature} \end{cases}$$

Formula for calculation

$$R.F.W. = \frac{Vt60 \times 1000}{\pi r^2 TP}, \text{ where } V = \text{vol. water in c.c.}$$

t = membrane thickness in mm.
r = membrane radius in cm.
T = time of flow of vol. V in sec.
P = water pressure producing flow in cm.

$$= K\frac{Vt}{T}, \text{ where } K \text{ is constant for standard conditions of measurement."}$$

Fig. 17 shows the form of cell used for these measurements.

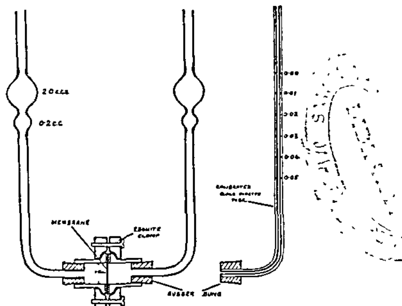


Fig. 17 Diagram showing construction of apparatus for estimating rate of water flow. It can be made by using two glass bottle necks suitably ground with carborundum.

(Reproduced from *J. Path. Bact.*, 1931, *34*, 505.)

For details regarding the estimation of S.W.C. see Elford and Ferry (1935), also Asheshov (1933).

Alternative Method

Elford's work has been successfully repeated by Bauer and Hughes (1935), working in America, who used a collodion product labelled Collodion X660–18.[1] These workers also prepared stock collodion solution, using Du Pont's nitrocellulose parlodion sheets, the exact composition of which was as follows:

Parlodion shreds, 150 gm., absolute alcohol, 250 gm., anhydrous ether, 750 gm., acetone, 1,150 gm., and primary amyl alcohol, 575 c.c.

Before use, parlodion sheets were well washed six times in distilled water, twice in 95 per cent. alcohol, and twice in absolute alcohol, then were used without drying. After the final washing, 250 gm. (316 c.c.) of absolute alcohol, which was freshly prepared by drying 95 per cent. alcohol over lime and distilling, was added to the parlodion shreds which were allowed to swell overnight. On the following

[1] Manufactured by Du Pont De Nemours of Parlin, New Jersey.

day, 750 gm. (1,043 c.c.) of anhydrous ether was added and the mixture shaken at intervals until the parlodion was dissolved. The solution was diluted with its own weight of acetone (1,453 c.c.) and agitated in a mechanical shaker for 2 hours. Primary amyl alcohol was then added in the proportions of 10 c.c. to each 40 gm. of the mixture and the solution again shaken for 2 hours. Finally, the stock preparation was allowed to stand for 2 to 3 weeks before being used. Bauer and Hughes stated that membranes manufactured with such parent collodion gave satisfactory results, and they also drew attention to the fact that the percentage of acetone and amyl alcohol used by them was not the same as that recommended by Elford (1931).

To prepare membranes the stock solution of collodion was diluted with an equal volume of a diluting mixture consisting of 1 part by weight of absolute alcohol and 9 parts of anhydrous ether. This diluted solution was measured in 200 c.c. amounts into a series of 6-ounce bottles fitted with screw caps lined with tin-foil.

Membranes made from such a solution possessed an A.P.D. of 0.6 to 0.8 μ, depending on the humidity and temperature at which evaporation of the solvents occurred. To make more permeable membranes, 2 c.c. of water was added to 200 c.c. of the diluted collodion and this yielded membranes of an A.P.D. 1.0 to 1.2 μ. For preparing membranes below 0.5 μ in A.P.D., glacial acetic acid of analytical quality was added, varying from 0.2 to 3.6 c.c. per 200 c.c. of diluted collodion solution. So many individual factors tended to affect the porosity of the membrane that Bauer and Hughes stated that no definite standards could be laid down, but in general they found that the addition of 1 c.c. of glacial acetic acid to 200 c.c. of collodion solution reduced the A.P.D. to 200 mμ, 2 c.c. reduced it to 100 mμ, and the addition of 3 c.c. reduced the A.P.D. still further to 15 mμ.

Further details were as follows. After addition of the acetic acid or water to the collodion, the mixture was shaken for 2 hours, the solution carefully poured into the cell, the contents of one bottle (200 c.c.) being used for a 40 cm. diameter cell. The optimum evaporation time was found to be 75 minutes, the temperature during evaporation 22° to 24° C., and the relative humidity from 60 to 65 per cent. After evaporation, to remove the membrane, the cell was immersed in water and, without exerting manual traction, the membrane permitted to free itself. The membranes, which were separated from each other by filter paper, were then washed in distilled water for 2 to 3 weeks and placed in a flat photographic developing tray. Disks were cut from the membranes and stored in distilled water in airtight jars; they were calibrated before use according to the method recommended by Elford (1931).

Types of Filter Suitable and Pressure Applied

In a personal communication, Dr. Elford has informed us that two varieties of

Either positive or negative pressure can be used, but when preparing stock filtrates of small amounts less than 20 c.c., or when employing relatively "tight membranes," it is usually more convenient to apply positive force. Thus to prepare a bacteria-free filtrate of a pathological fluid (well-diluted and clarified), a membrane of A.P.D. 700 to 750 mμ should be used, with either a positive or a negative pressure of 25 cm. of mercury.

The Interpretation of the Results of Ultrafiltration

Like other forms of filter, the same factors govern the passage of particles through collodion membranes, but by virtue of their thinness and uniformity of porosity, many of the inherent disadvantages of earthenware filters have been

eliminated The properties of these membranes have been investigated with meticulous care by Elford (1933) who has inquired into the slight effects due to adsorption, variations in the thickness of individual membranes, and the influence of different pressures After consideration of the mode by which each of these individual factors operates Elford (1933) has been able to define the optimum conditions for filtration For example, a 48-hours' old agar culture of *B. prodigiosus* suspended in broth at pH 7 6 was retained by all membranes of pore size less than

0 75 μ. The same effect was demonstrated in the case of the organism of pleuropneumonia, a 3-days' old serum broth culture at 37° C. containing particulate spherical bodies of 0 2 μ to 0 5 μ in size, together with invisible phases below this limit. By filtering the culture through membranes of graded porosity, it was shown that the larger visible spheres failed to pass membranes below 0 35 μ A.P.D., whereas the smaller forms did A membrane of 0 35 μ therefore retained particles of 0 2 to 0 25 μ in size.

It was also pointed out that the concentration of bacteria in the initial bacillary suspension largely determined the number of them which entered the filtrate, an analogous observation was made in the case of foot-and-mouth disease virus by Galloway and Elford (1931). As filtration proceeds the pores of the membrane tend to become progressively clogged, until finally an end-point is reached at which the forces of adsorption and cohesion exert an abnormal effect and slow the rate of filtration The filtration end-point may thus be experimentally ascertained and plotted graphically.

The results obtained with Elford's (1933) membranes showed that the first falling off in maximum filtrate concentration usually occurs at a pore size value varying from two to three times the absolute limiting value for nonfiltrability Fig. 18 shows a graph indicating the filtration characteristics of *B prodigiosus* and vaccinia virus when compared.

The sizes of viruses as determined by ultrafiltration are in close agreement with those ascertained by ultraviolet light photomicrography. For example, ectromelia virus was estimated to measure 0 1 to 0.15 μ by filtration and 0 13 to 0 14 μ by micrometry, according to Barnard and Elford (1931). Likewise, Elford and Andrewes (1932) calculated that the elementary bodies of vaccinia measured 0 125 to 0.175 μ by filtration, and Barnard found them to be 0.17 to 0 18 μ by ultraviolet

Dawson and McFarlane
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Estimation of particle size.

From the results of filtration end-point tests obtained under the optimum experimental conditions, Table 2 indicates the probable size of the smallest particle just retained by a given membrane.

TABLE 2

Membrane *Average pore diameter*	*Size of particle retained*
mμ	
10–100	(0 33–0 5) *d*
100–500	(0 5–0 75) *d*
500–1,000	(0 75–1 0) *d*

d = the average pore diameter of limiting membrane for optimum filtration conditions

day, 750 gm (1,043 c.c.) of anhydrous ether was added and the mixture shaken at intervals until the parlodion was dissolved. The solution was diluted with its own weight of acetone (1,453 c.c.) and agitated in a mechanical shaker for 2 hours Primary amyl alcohol was then added in the proportions of 10 c.c. to each 40 gm. of the mixture and the solution again shaken for 2 hours. Finally, the stock preparation was allowed to stand for 2 to 3 weeks before being used Bauer and Hughes stated that membranes manufactured with such parent collodion gave satisfactory results, and they also drew attention to the fact that the percentage of acetone and amyl alcohol used by them was not the same as that recommended by Elford (1931).

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It was also pointed out that the concentration of bacteria in the initial bacillary suspension largely determined the *number* of them which *entered the filtrate, an* analogous observation was made in the case of foot-and-mouth disease virus by Galloway and Elford (1931) As filtration proceeds the pores of the membrane tend to become progressively clogged, until finally an end-point is reached at which the forces of adsorption and cohesion exert an abnormal effect and slow the rate of filtration The filtration end-point may thus be experimentally ascertained and plotted graphically.

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500–1,000	(0 75–1 0) *d*

d = the average pore diameter *of limiting membrane for optimum filtration conditions*

(Additional details regarding the construction of collodion membranes are dealt with by Elford, Grabar, and Ferry, 1935.) However, Elford (1933) has drawn attention to the fact that the number of particles (e g., *B prodigiosus*) present in a filtrate is dependent upon their concentration in the initial suspension, and Galloway and Elford (1931) have also referred to the same point in connection with foot-and-mouth disease virus This criticism of the value of ultrafiltration as a method for measuring the size of viruses has also been brought forward by Levaditi *et al* (1936), who drew attention to certain pitfalls in the use of ultrafiltration methods. Working with lymphogranuloma inguinale virus, they obtained different sizes for the virus by employing tissue suspensions of varying degrees of pathogenicity. Thus, from filtering a feebly active tissue extract they calculated its size to be 600 to 800 mμ, but by using a more active suspension they found it to be 250 to 350 mμ, and by employing a highly virulent preparation they estimated the size of the virus to measure 100 to 140 mμ. From these observations, it was con-

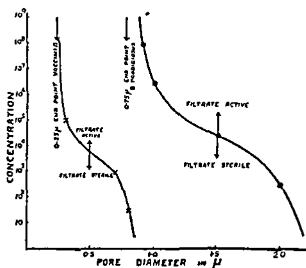


FIG. 18. Graph showing behavior of vaccinia virus and *B prodigiosus* when subjected to ultrafiltration

(Reproduced from *Brit J exp Path*, 1932, *13*, 36)

cluded that the more actively pathogenic was the virus material, the smaller appeared to be its size

Levaditi *et al* (1936) also stressed a second factor liable to influence the results, namely, the susceptibility of the species of animal inoculated with the filtrate when the latter is tested for pathogenicity For example, when filtrates of lymphogranuloma inguinale tissue extracts were injected into a relatively insusceptible animal such as a mouse, they found that the virus appeared to measure 430 to 507 mμ, but if a more susceptible animal such as a monkey was used, then it was found that the virus measured 120 to 180 mμ in size.

Similar deductions were made earlier by Levaditi and Nicolau (1923) in connection with the filtration of neurotropic viruses through collodion sacs The French workers have accordingly concluded that the two factors mentioned have an important bearing on the problem and should be taken into account when measuring the size of viruses by ultrafiltration methods.

These observations are of great interest and suggest that further work on this aspect of ultrafiltration studies is necessary.

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CHAPTER VI

THE CENTRIFUGALIZATION OF ELEMENTARY BODIES

In principle, the act of centrifugalizing elementary bodies is no different from that of spinning down bacteria in the ordinary way. A much higher speed is, however, usually demanded, for owing to the smaller size of elementary bodies a greater force is required to deposit them.

The researches of Ledingham (1931) demonstrated that the elementary bodies of fowl-pox (and vaccinia) could be deposited by centrifugalization at 12,000 revolutions per minute (r p m) for 1 hour, after which the supernatant fluid lost its infectivity and the deposited material consisted of innumerable elementary bodies Suspensions of elementary bodies prepared in this manner were agglutinated by specific antisera, and thus their etiological relationship to fowl-pox was proved beyond all doubt. Amies (1934) used the same technique for centrifugalizing the elementary bodies of zoster and varicella, these were obtained by diluting vesicle fluid in 0 86 per cent. saline containing 2 per cent. sodium citrate, centrifugalizing at low speed to throw down tissue cells, and thereafter spinning at 12,000 r p m for 30 minutes to deposit the elementary bodies Such an antigen consisting of a suspension of elementary bodies was used by Amies (1934) for agglutination tests with the sera of patients who had recovered from zoster and varicella infections, respectively.

Both Ledingham and Amies used the bucket type of centrifuge, but other varieties of instrument have also been employed with greater success and some of these are referred to below

TYPES OF CENTRIFUGE

The majority of workers has employed machines of the high-speed type, but Craigie (1932) has shown that they are not essential, since elementary bodies can be deposited at a comparatively low speed provided that the instrument is of the angle design

Centrifuges in common use at the present time may be divided into four classes, namely

1. Those fitted with a *bucket type* of rotating head, driven by a high-speed electric motor such as in the Ecco machine, or by a slower speed motor operating via a geared pulley similar to the old type employed by Ledingham (1931)

2 *The angle centrifuge* This is fitted with a slow-speed motor but is, nevertheless, capable of depositing elementary bodies by virtue of the fact that the construction of the head and the narrowness of the flat glass tube employed compensate for the loss of speed

3. *The Sharples centrifuge* This consists of a cylinder rotating vertically in its long axis at 50,000 r p m into which material is introduced, and propelled by an electric motor, compressed air or steam turbine.

4 *Air-driven instruments* These are based on the principle devised by Henriot and Huguenard (1925), being propelled by a current of compressed air which strikes the slots of an air turbine that can be made to revolve at 60,000 r p m

1. Centrifuges fitted with the Bucket Type of Head and driven by a directly coupled or geared electric motor

The Ecco centrifuge (bucket type).

Both Elford (1936) and we ourselves (van Rooyen and Rhodes, 1937) have used such an instrument This is fitted with a direct drive (d c) electric motor

which revolves at 15,000 r p m and carries four buckets which accommodate hard

(1936) states that the motor attains 4,000 r p m. in under a minute and 10,000 r.p.m. in just over a minute It also slows down quickly and stops within a minute when turning at 4,000 r p m and in one and a half minutes from 10,000 r p m

Measuring the speed of the centrifuge.

By placing a revolution counter or tachometer on the end of the revolving spindle and timing the results with a stopwatch, the speed of the instrument can be calculated At a high speed such as 15,000 r p m this method is probably not the best, for owing to unavoidable losses due to slip and other effects the results tend to be inaccurate Elford (1936) has devised a clever optical contrivance for estimating accurately the speed of the motor, and his description of it is as follows

"A cylindrical tubular extension was attached to the screw cap which fitted the spindle of the centrifuge, projecting about 2 cm. above the top of the metal case. A hole was drilled through this tube at right angles to its axis Light from a pointolite lamp was gathered by a lens and directed as a narrow beam through the hole in the centrifuge extension tube on to the periphery of a toothed disk which could be rotated at a controlled speed The teeth were viewed in a telescope set in alignment with the beam of

r p m The speed of the disk was regulated by means of a potentiometer resistance in series with a small universal motor and was measured by counting the revolutions with the aid of a speedometer and stop-watch In order to embrace the complete range of speeds up to 15,000 r p m two disks each having 72 teeth were coupled by gears in the ratio 1 7 5

"This method enables the speed to be measured without affecting the centrifuge in any way, and is therefore to be preferred to the use of a tachometer."

2. Centrifuges fitted with the Angle Type of Head and driven by directly coupled slow-running electric motor

The ordinary laboratory type of instrument (Bolaget) has been employed by Craigie (1932) The success of this machine depends on the fact that flat glass tubes are used, which possess an internal width of 4 to 5 mm, and consequently the elementary bodies have only to traverse a short distance before they strike the lateral wall of the tube which is placed at an angle of 28° from the vertical With such a centrifuge a speed of 3,500 r p m for 60 minutes was found to be sufficient for depositing the Paschen bodies of vaccinia

An angle centrifuge fitted with a high-speed motor giving a speed of 18,000 r p m and 34–800 times gravity is also obtainable and better results may be expected with this instrument (Pickels, 1942)

3. The Sharples Centrifuge

The closed bowl technique (Schlesinger, 1936)

This ingenious principle makes it possible to obtain a centrifugal force of 20,000 × gravity and provides a quick method of sedimenting viruses To operate the instrument, the inner wall of the rotating cylinder is first coated with a thin layer of agar, which is allowed to solidify, and a small amount of virus suspension is placed inside the cylinder The latter has an internal radius of 2 cm, is 20 cm. high, and revolves at 32,000 r p m Thus when turning at high velocity, the fluid within becomes evenly distributed as a thin film over the surface of the agar, so

CHAPTER VI

THE CENTRIFUGALIZATION OF ELEMENTARY BODIES

In principle, the act of ... lifferent from that of spinning down bacter ... speed is, however, usually demanded, for ... bodies a greater force is required to deposit them.

The researches of Ledingham (1931) demonstrated that the elementary bodies of fowl-pox (and vaccinia) could be deposited by centrifugalization at 12,000 revolutions per minute (r.p.m.) for 1 hour, after which the supernatant fluid lost its infectivity and the deposited material consisted of innumerable elementary bodies. Suspensions of elementary bodies prepared in this manner were agglutinated by specific antisera, and thus their etiological relationship to fowl-pox was proved beyond all doubt. Amies (1934) used the same technique for centrifugalizing the elementary bodies of zoster and varicella, these were obtained by diluting vesicle fluid in 0 86 per cent. saline containing 2 per cent. sodium citrate, centrifugalizing at low speed to throw down tissue cells, and thereafter spinning at 12,000 r p m. for 30 minutes to deposit the elementary bodies. Such an antigen consisting of a suspension of elementary bodies was used by Amies (1934) for agglutination tests with the sera of patients who had recovered from zoster and varicella infections, respectively.

Both Ledingham and Amies used the bucket type of centrifuge, but other varieties of instrument have also been employed with greater success and some of these are referred to below.

TYPES OF CENTRIFUGE

The majority of workers has employed machines of the high-speed type, but Craigie (1932) has shown that they are not essential, since elementary bodies can be deposited at a comparatively low speed provided that the instrument is of the angle design

Centrifuges in common use at the present time may be divided into four classes, namely

1. *Those fitted with a bucket type of rotating head, driven by a high-speed* electric motor such as in the Ecco machine, or by a slower speed motor operating via a geared pulley similar to the old type employed by Ledingham (1931).

2 *The angle centrifuge* This is fitted with a slow-speed motor but is, nevertheless, capable of depositing elementary bodies by virtue of the fact that the construction of the head and the narrowness of the flat glass tube employed compensate for the loss of speed

3. *The Sharples centrifuge* This consists of a cylinder rotating vertically in its long axis at 50,000 r p m into which material is introduced, and propelled by an electric motor, compressed air or steam turbine

4. *Air-driven instruments* These are based on the principle devised by Henriot and Huguenard (1925), being propelled by a current of compressed air which strikes the slots of an air turbine that can be made to revolve at 60,000 r.p m.

1. Centrifuges fitted with the Bucket Type of Head and driven by a directly coupled or geared electric motor

The Ecco centrifuge (bucket type).

Both Elford (1936) and we ourselves (van Rooyen and Rhodes, 1937) have used such an instrument This is fitted with a direct drive (d c) electric motor

The rotor (A) consists of an inverted duralumin cone of 7.6 cm. diameter at its base with a vertex angle of 110°. The top of the base (B) is flat and has four radial recesses cut into it capable of taking four small tubes 2 by 0.5 cm. in size. The tubes are held down during spinning by a flat duralumin disk (C) which is screwed (D) down on the top of the cone. At the foot of the under surface of the rotor a series of cuts or grooves is made and arranged, either in a radial or helicoidal manner, so as to assist the driving force of the air after the principle of an air turbine.

The stator (E). This is a hollow cone made of phosphor bronze provided with nine holes round its interior (I), through which compressed air is forced and allowed to impinge upon the grooves cut into the sides of the rotor cone in an upward and anticlockwise direction. The angle of the stator is 90° to 92°, and the air-holes or jets should be drilled down (F) about 2 cm. from the upper and inner margin of the cone, at an angle of 45° to the axis of the cone when viewed antero-posteriorly and vertically when viewed from the side. The running of the instrument is controlled by an adjustable valve (G), and at the conclusion of the experiment the spinning rotor is lifted off the stator with a metal fork.

Compression plant. To maintain a speed of 60,000 r.p.m., a compressed air plant driven by a 4-h.p. electric motor, and delivering 20 cubic feet per minute at a pressure of 80 to 90 lb. per sq. inch, is required.

Device for estimating speed of centrifuge. McIntosh and Selbie (1937) have used a stroboscope for calculating the speed of their air-driven centrifuge. The method involves fitting up a neon lamp, possessing a frequency of 100 cycles per second, that is worked by an electrically driven tuning-fork yielding a frequency of 6,000 cycles per minute.

A V-shaped mark is made in the revolving top of the centrifuge and the neon lamp is held above it while it spins. When the instrument reaches 6,000 r.p.m. the V-shaped mark appears stationary, and also at each subsequent multiple of 6,000 r.p.m. Thus a speed of 60,000 r.p.m. is reached after the V mark has become stationary ten times. Halves and quarters of these figures can also be calculated, as two V's are visible at half a multiple and four at a quarter of a multiple.

Beams and Pickels (1935) designed an instrument which operated at 100,000 r.p.m., and by the use of hydrogen gas a rotational speed of 1,200,000 r.p.m. was reported earlier by Beams, Pickels, and Weed (1934). Unfortunately, the results of sedimentation experiments with such machines were found to be inaccurate because the thickness of the solution layer had to be limited to 0.1 cm. or less, and undesirable convection currents were introduced through overheating of the rotor (Bauer and Pickels, 1937).

The Svedberg centrifuge.

This is the fastest practical type of centrifuge that has been constructed. The instrument is driven by oil turbines, and a speed of 160,000 r.p.m. has been obtained. The rotor weighs 12.5 gm., the cell 12.9 gm., and for sedimentation measurements up to 900,000 times gravity the two twin turbines operating at each end of the shaft are fed with oil at a pressure of 15 Kg./cm., and during rotation an atmosphere of 20 mm. of hydrogen is maintained. With such an instrument, Svedberg, Boestad, and Eriksson-Quensel (1934) have measured the sedimentation rate of dilute hemoglobin.

Optical ultracentrifuges of the vacuum pattern.

Owing to their great cost of manufacture the oil-driven Svedberg precision centrifuges have been beyond the reach of many virus workers, and less costly machines have been constructed to operate with compressed air power.

Pickels and Beams (1935) devised one of the latter variety, propelled by a compressed air turbine of the spinning-top type, attached by a length of piano

that the virus particles have only a very short distance to traverse before they impinge upon and adhere to the surface of the agar.

The interior of the bowl is coated with agar by first warming the cylinder, introducing melted agar into it, and allowing it to solidify while the cylinder is in motion. For particles less than 20 mμ in diameter 2 per cent. agar is used, and for viruses less than 60 mμ in diameter 1 per cent. agar.

The latest design of the Sharples centrifuge, the Presurtite model, is driven by either compressed air or steam turbine at 50,000 r.p.m. and with a force of 62,000 × gravity. The steam model can be fitted with refrigeration coils to stop overheating. Four sizes of Sharples centrifuge bowls are available, the standard pattern has a volume of 250 c.c. with a layer of residual liquid ¾" deep, and the others have layers of ½", ¼" and ⅛", respectively, with lesser capacity. The highest efficiency is obtained with the ⅛", ¼" and ½" liquid layer bowls, and influenza virus can readily be sedimented without the use of agar lining (Stanley, 1946). Even poliomyelitis virus has been deposited with this type of centrifuge (Melnick, 1946).

4. Air-driven Centrifuges

McIntosh and Selbie's centrifuge.

McIntosh and Selbie (1937) have constructed a high-speed instrument, based on the design of the Henriot-Huguenard (1925) machine, which has no metal bearings and superficially resembles a large humming-top. It is driven by a current

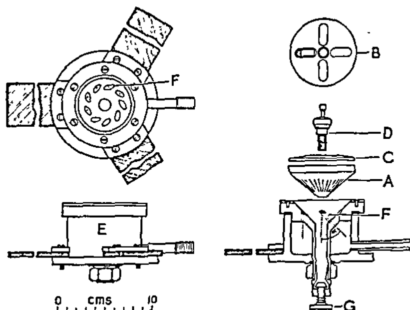


Fig. 19 Sectional diagram showing design of air-driven humming top centrifuge

(Reproduced from *Brit. J. exp. Path.*, 1937, *18*, 162.)

of compressed air which impinges upon the bottom of its outer casing into which a number of slots is cut. The machine therefore revolves on a cushion of air, and any loss of speed arising from friction due to bearings is eliminated. Four small tubes measuring 2 by 0.5 cm. are carried in narrow recesses on the top of the rotor and a speed of 60,000 r.p.m. can be obtained. Fig. 19 indicates the constructional details of the apparatus.

(B). A steel shaft of piano wire 0.100 to 0.110 in. in diameter capable of withstanding 300 to 900 revolutions per second (r.p.s.). For speeds less than 300 r.p.s. a piece of 3/16 in drill rod should be used instead, in order to eliminate vibration

(N). A mild steel conical plate containing a number of holes acting as ports through which air is forced from inlet (M) at 100 lb. per sq in., requisite for a speed of 900 r.p.s. (see Beams and Pickels, 1935).

(L) is the air inlet referred to as the passage for introducing (supporting pressure) air at 30 to 40 lb per sq in used for slow running at 200 r.p.s

(K) and (J) are inlets for oil supplied at a pressure of 5 to 15 lb. per sq in.

g Oil-seal bearing

q. Oil-well.

(r). Oil-drain.

i Conical deflector for oil supply.

e. A short length of rubber pressure tubing

n. Rotor clutch.

c. Rotor made of duralumin alloy 14 S.T., 7 in in diameter, of tapering thickness, being 1 in at periphery and 2 in. at center The outside edge of the cell hole is ½ in from the periphery of the rotor, and the distance between the center of the cell (w) and axis of rotation is 6.5 cm

w. Cell for holding suspension to be centrifuged Is similar to that of Svedberg's. Its main difference is that, being filled from the end during assembly, an oil-seal is unnecessary.

u Dummy cell for balancing rotor

d is the top of the centrifuge housing made of ½ in boiler plate into which the upper of the two quartz glass windows is fitted This is not shown in the sectional diagram The remainder of the rotor is housed in an airtight casing constructed of chrom-vanadium steel, and while it revolves a vacuum of less than 1 mm of mercury is maintained in the chamber by a quick-operating oil pump

The optical system used for [illegible] within the cell during centrifugalizati[illegible] from a mercury vapor arc lamp are r[illegible] lenses, passed through bromine and [illegible] mirror through quartz glass windows placed in the bottom of the vacuum chamber, through the cell (every time the apertures register during rotation), through the top quartz window fitted in (d), reflected by a second aluminum mirror, and finally focused by a 100 cm focal length quartz-fluorite lens upon a photographic plate on which the image is recorded The total distance from the center of the [illegible]

[illegible] used consists of watching a spot placed on the top of the turbine (A) through a slotted disk that is mounted, together with a small magneto, on the shaft of an electric motor The speed of the centrifuge is thus calculated directly in terms of the voltage developed by the magneto At 900 r.p.s (or 60×900 r.p.m.) the centrifugal force at the center of the cell is estimated to be 225,000 times gravity, and at 800 r.p.s (or 48,000 r.p.m.) the force is 180,000 times gravity

Heating during running After some hours of continuous use, the maximum rise of temperature for 800 r.p.s. is found to be 2° C.

Another modification has been introduced by Beams and Black (1939) and Skarstrom and Beams (1940) who replaced compressed air by a high-speed electrical drive, in which the weight of the rotor was supported electromagnetically.

The Size of Viruses Demonstrated by Centrifugalization

The centrifuge designed by Svedberg was fitted with specially constructed windows which allowed optical observations to be made during centrifugation and

wire to the rotor housed in a vacuum chamber. Bauer and Pickels (1936–7) carried out further improvements and successfully sedimented egg albumin at 60,000 r p m. at a centrifugal force of 260,000 times gravity.

Biscoe, Pickels, and Wyckoff's (1936) modification of Svedberg's centrifuge. These workers have designed an air-driven machine with which they have been able to measure optically the sedimentation rate of crystalline proteins of the tobacco mosaic virus. The instrument embodies many of the principles incorporated in Svedberg's centrifuge, and in particular the optical device by which it is possible to watch the sedimentation rate of particles during centrifugalization

The rotor of the machine runs in a vacuum and is enclosed inside an airtight metal casing. Two quartz glass windows are fitted opposite to each other at the top and bottom of this covering, and a beam of ultraviolet light is passed through these so that the progress of sedimentation may be recorded photographically from time to time. For details regarding the fitting of the optical equipment, the reader is referred to the work of Svedberg (1934), Svedberg and Nichols (1927), and Svedberg, Boestad, and Eriksson-Quensel (1934).

Fig. 20 indicates the propelling mechanism of the Biscoe-Pickels-Wyckoff centrifuge seen in cross-section, and its operation is as follows A current of air under pressure enters at either port (L) or (M), passes upward and impinges against

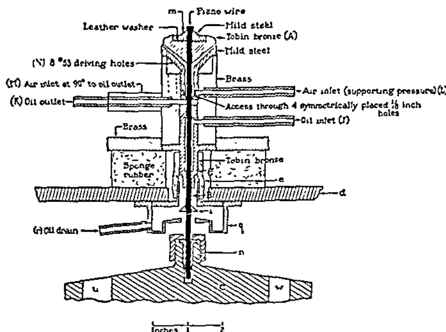


Fig 20 Cross-sectional drawing of the rotating system of the air-driven turbine centrifuge designed by Biscoe, Pickels, and Wyckoff (1936)
(Reproduced from *J exp Med*, 1936, *66*, 39)

grooves cut into the sides of a mobile bronze turbine (A) so that this is set in motion The latter is attached to the rotor (C) of the centrifuge by means of a flexible shaft (B) which revolves simultaneously. Additional constructional details are as follows

(A). Bronze air-driven turbine, the conical surfaces of which have grooves cut into them For details regarding the dimensions, number, and position of these milled flutings, see Beams and Pickels (1935)

m Leather washer acting as a turbine clutch

= initial concentration, and C_t = the concentration after spinning for time t. For conditions such that $C_t/C_0 = 0.1$ this relationship becomes

$$d = k\sqrt{\left(\frac{\eta}{(\sigma_p - \sigma_m)N^2 t}\right)},$$

where $k = 1.78 \times 10^7$ for the metal cells, or 1.84×10^7 for glass cells

By employing this principle for measurement, Elford and Andrewes (1936) found that vaccinia virus measured 170 to 180 mμ, influenza virus 87 to 99 mμ, and the Rous sarcoma virus 60 to 70 mμ.

Elford and Galloway (1937), using this inverted capillary tube technique, found that the size of foot-and-mouth virus was 20 mμ, assuming that the density of the particles was 1.30. Different strains of foot-and-mouth virus appeared to be of the same size and moreover their particles tended to be relatively uniform in dimension.

The size of vesicular stomatitis virus was also measured by the same technique, but its particle size tended to vary and ranged from 74 to 60 mμ, according to whether the density of the particle was assumed to be 1.20 or 1 30, respectively. Subsequent studies by Tang, Elford, and Galloway (1937) showed that equine encephalomyelitis virus measured 39 to 32 mμ, louping-ill virus 27 to 22 mμ, and a *B megatherium* bacteriophage 30 to 37 mμ. The need for more precise knowledge regarding the densities of the particles was emphasized by these workers.

McIntosh and Selbie (1937) carried out similar methods of measurement by using their air-driven centrifuge constructed on the lines of the Henriot and Huguenard (1925) instrument, capable of attaining a speed of 60,000 revolutions per minute. The sedimentation rate of various bodies was expressed graphically to show "a sedimentation angle," and the size of the particle was proved to be proportional to the square root of that angle, i e., $D = K \times \tan\theta$, where D = the diameter of the particle, K = the speed of the centrifuge, and θ = the sedimentation angle.

These workers have used this simple formula for determining the size of virus particles, and have drawn attention to the similarity of their formula to that of Stokes. From their results, they estimated that vaccinia virus measured 99 to 220 mμ from the sedimentation angle, and 103 to 240 mμ from their modification of Stokes' equation.

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thus record the rate of sedimentation. Bechhold and Schlesinger (1931) employed the ordinary bucket type of high-speed centrifuge for similar studies, and later Schlesinger (1932) used the same method for calculating the size of viruses and bacteriophages. In his experiments, Schlesinger centrifuged the virus suspension in a wide flat-bottomed tube 5 cm. long and 1 cm. broad, containing at its foot a thick disk of filter paper the purpose of which was to minimize dispersion of the deposited virus particles after centrifugalization. The infectivity-titer of the fluid was first ascertained before centrifugalization (C_0) and later compared with the loss in titer after centrifugalization (C_t), from which he was able to deduce the probable size of the virus particles (*d*) by application of the following formula:

$$d = 6.15 \times 10^{9} \sqrt{\left(\frac{\eta b \log C_0 \times C_t}{(\sigma_p - \sigma_m) R T N^2}\right)},$$

where d = the diameter of the particles in *m*μ, η = *the viscosity of the medium* in C.G.S. units, σ_p = the density of the particle, σ_m = the density of the medium, b = the height of the column of liquid, R = the distance of the filter paper from the axis of rotation, N = the r.p.m. of the centrifuge, and T = the time of centrifugalization in minutes. The above formula has been based on a series of experiments made by Schlesinger (1932) on the size of a range of gold sols of known dimensions.

The specific gravity of a virus is best calculated by centrifuging suspensions made up in varying densities of saccharose solution, to determine the lowest concentration of sugar solution in which the virus fails to be deposited. For example, Schlesinger and Andrewes (1937), using the Sharples centrifuge, found that after *spinning Shope's fibroma virus for 75 minutes at 33,000 r.p.m., there was just de*tectable deposition in a medium of specific gravity 1.25. They therefore assumed that the specific gravity of the virus was 1.3, and from the results of their calculations concluded that the fibroma virus measured 126 to 141 mμ, and the papilloma virus 32 to 50 mμ. These figures closely approximated to those obtained by ultrafiltration, from which it was learned that the fibroma virus measured 125 to 175 mμ, and the papilloma agent 23 to 35 mμ in diameter.

Although the sizes of viruses and bacteria measured by Schlesinger's technique were found to be in good agreement, they invariably appeared to be about 30 per cent. higher than the values obtained by ultraviolet light and ultrafiltration, and accordingly Elford (1936) reinvestigated the problem and introduced the inverted *capillary tube technique which overcame certain disadvantages associated with* Schlesinger's (1932) technique.

Elford's (1936) improved method greatly facilitated the obtaining of samples of material after centrifugalization, and minimized the possibility of redispersal of the virus particles after their deposition. It also enabled the investigator to compare the difference in infectivity of the fluid within the cell before and after centrifugalization, from the results of which it was possible to calculate the size of the virus particles (*d*) by direct application of Stokes' law, the following formula being used:

$$d = 7.94 \times 10 \sqrt{\left(\frac{\eta \log \frac{x_1 + l}{x_1 + lC_t/C_0}}{(\sigma_p - \sigma_m) N^2 t}\right)},$$

where d = the diameter of the particle in mμ, x_1 = the distance of the top of the capillary from the center of rotation (= 7.25 cm. for metal cell or 6.75 cm. for glass cell), l = depth of inverted capillary tube (= 1 cm.), σ_p = the density of the particle, σ_m = the density *of the medium*, η = *the viscosity of the medium*, N = the speed of rotation in r.p.m., t = the time of centrifugalization in minutes, C_0

CHAPTER VII

SOME PHYSICO-CHEMICAL PROPERTIES OF MAMMALIAN VIRUSES

The Sedimentation Constant and Molecular Weight

Svedberg and Nichols (1923) demonstrated that the rate of sedimentation of gold sols in a convection free cell could be measured quantitatively in a specially con-

and Lauffer (1943).

The following are some of the many factors which enter calculations to find the sedimentation rate and molecular weight of particles. molecular weight (M), angular velocity in radians per second (ω), distance from axis of rotation (x), density of the medium (ρ), density of the particle (σ), partial specific volume of the particle (V), molar frictional sedimentation coefficient (f_s), frictional coefficient of unsolvated and spherically shaped particle (f_0), viscosity of the medium (η), sedimentation velocity per unit field of force (s), time (t), diffusion constant (D), molar frictional diffusion constant (f_D), gas constant (R), absolute temperature in degrees absolute (T), radius of spherical particle (r), concentration at any time (C_t) and at original time (C_0), radial distance to meniscus is (x_0) and to boundary

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tions should be dilute, the particles electrically neutral and large in comparison with those of the surrounding medium.

The net centrifugal force acting on a mole of particles at a distance x from the axis of rotation under these conditions is the difference between their centrifugal *weight and the buoyancy exerted by the displaced medium This force is exactly* opposed by a frictional force which depends only on the velocity of sedimentation and a frictional coefficient Thus

$$M(1 - V\rho)\ \omega^2 x = f_s \frac{dx}{dt}. \qquad (1)$$

For dilute solutions a relationship exists between the frictional coefficient effective in diffusion and the diffusion constant,

$$f_D = RT/D, \qquad (2)$$

It has been found experimentally that under a given set of conditions the frictional coefficient effective in sedimentation and that operating in diffusion are equal The frictional coefficients can therefore be eliminated from the above equations and the well-known Svedberg expression is obtained

$$M = \frac{RTs}{D(1 - V\rho)} \qquad (3)$$

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The experimental value, f_s, of the frictional coefficient can be determined for a given particle from equation (1) Using the same values of M and V, f_0 can be calculated for the same particle from equation (4), i e, the frictional coefficient the particle would have if it were spherical and unhydrated. The ratio f_s/f_0, known as the frictional ratio, must be equal to or greater than unity.

If f_s/f_0 is equal to 1 0 for a given substance, its molecules must be of compact spherical shape and unhydrated If the ratio exceeds unity it can be said that the molecule is either hydrated or departs from spherical shape—or more probably both A good deal of further information is necessary before it is possible to say how much of the excessive frictional ratio is due to hydration and how much is due to asymmetry A number of protein molecules, however, have $f_s/f_0 < 1.13$ it seems that these molecules might be spherical, since such a frictional ratio would be obtained if a spherical molecule were hydrated to the extent of 30 per cent, an entirely plausible value Many proteins have much larger frictional ratios which cannot be explained as due to hydration alone, and these molecules probably deviate very markedly from spherical shape Further data on asymmetry and hydration of protein particles are given by measurement of stream birefringence, viscosity, and dielectric dispersion This subject is discussed at length by Cohn and Edsall (1943).

Optical Methods for Determining Sedimentation Rate of Viruses

Svedberg and Rinde (1924) demonstrated that if a beam of monochromatic filtered ultraviolet light of fixed wave length emitted from a mercury arc lamp was passed through the cell of ould absorb it, whereas the solvent to obtain a series of photographs ary at successive intervals Later, in which the position of the sedimentation boundary could be ascertained by measuring the deflection of light rays through regions of sharp variation in refractive index such as were present at a sedimenting boundary According to Pickels (1944), a good refractive index system is subject to fewer errors than an absorption system, and is more suitable for the study of mixtures where it is desired to learn the relative concentrations of individual components In contrast, greater proportional variation occurs in the absorptive coefficients of proteins in the ultraviolet region than exist in specific refractive indices of the visible band (McFarlane, 1935). The absorption method possesses certain advantages such as, for example, in the case of yellow fever virus, when present in infected monkey serum, this virus displays high differential absorption in the 320–440 mμ spectral region and cannot be demonstrated by the refractive index system (Pickels and Bauer, 1940).

The Sedimentation Boundary (Sedimentation Properties)

Studies on plant viruses have shown that in the case of bushy stunt virus, the particles are strictly homogeneous in size, shape, and density, resembling the molecules of a typical protein (Lauffer, 1942) Similar investigations on tobacco mosaic virus demonstrated that the agent was a rod-shaped structure measuring 15 × 275 mμ, that possessed the high molecular weight of 4×10^7 (Knight and Stanley, 1941) Further ultracentrifugation studies revealed that the tobacco mosaic virus could be rapidly disintegrated by 6M urea and 0 1 M phosphate buffer at pH 7, following which low molecular weight components appeared that were devoid of nucleic acid, did not show double refraction of flow, and were insoluble in dilute buffers and lacked the property of infectivity

In the field of human and animal virus diseases, ultracentrifugation studies have encountered varying degrees of success The Rous sarcoma agent was found to lack the property of double refraction or anisotrophy of flow so frequently observed in the case of pure plant virus proteins, the Rous agent possessed a sedi-

The sedimentation constant, $s = \frac{dx/dt}{\omega^2 x}$, is the rate of sedimentation in unit centrifugal field, and is a characteristic constant for a given molecular species under specified conditions. In order to compare sedimentation constants obtained in different solutions at different temperatures it has been suggested by Svedberg that they should be reduced to the same standard conditions Such a reduced sedimen- mentation the materials would have if dissolved

ally given in Svedberg units, i e., in units of centimeters per sec. per unit field of force multiplied by 10^{-13}. The sedimentation of hemoglobin, for example, can be expressed in the following equivalent ways

$$\begin{aligned} s_{20} &= 4\,4 \times 10^{-13} \text{ cm/sec/dyne/gm} \\ &= 4\,4 \times 10^{-13} \text{ cm/sec/unit field of force} \\ &= 4\,4 \times 10^{-13} \text{ sec (absolute units)} \\ &= 4\,4\,\text{S} \quad \text{(Svedberg units).} \end{aligned}$$

The Svedberg formula is derived without making any assumptions about the shape and structure of the particle. Molecular or particle weights calculated from this equation should, therefore, be equally free from assumptions The molecular weight M is seen to depend on s, D, V, ρ, quantities which can readily be measured experimentally.

The Effect of Electrical Charge, Density, Hydration, and Shape on Sedimentation Rate

The Svedberg equation is derived under the condition that the particles examined should be electrically neutral with respect to the medium Otherwise, an abnormal sedimentation rate is obtained owing to the resulting Donnan effect, the sedimentation of the heavier colloidal ion is retarded by the electrostatic influence *of the lighter ions In practice this effect is eliminated by the addition of a non-*sedimenting electrolyte, 0 2 M NaCl or KCl is usually sufficient for 1 per cent protein solution

The partial specific volume (V), or reciprocal dry density ($1/\sigma$), is usually determined by the pyknometer method, in which the volume displaced by a known weight of substance is measured The concentration of the solution used is determined by estimating *the dry weight of solute in the solution and in this measure-*ment some uncertainty enters since many of the solutes remain solvent tenaciously, making precise estimation of the dry weight difficult The partial specific volume as determined by this method, however, may not represent the effective partial specific volume of the same solute under actual conditions of sedimentation because of the possibility of hydration of the particle Such a change in the properties of the solute would be represented by an increase in the *frictional coefficient* f_s *of* equation (1) The net centrifugal force is changed but slightly since any increase in the centrifugal weight of the particle is opposed by the increased buoyancy of the medium Any change in f_s due to hydration results in a similar change in f_D and, since the frictional coefficients are eliminated from equations (1) and (2) to obtain the Svedberg expression (3), M as calculated from this latter equation is the weight of the particle *in the unsolvated state to a close approximation, provided* V is the value for the dried material This problem is treated mathematically by Kraemer (Svedberg, 1940), and in simple physical terms by Pickels (1942)

A compact spherical, unsolvated particle sedimenting in the ultracentrifuge will have the minimum frictional coefficient f_o given by Stokes' equation

$$(4) \qquad f_o = 6\pi\eta N \left(\frac{3MV}{4\pi N}\right)^{1/3}$$

fluenza A (PR8). Sharp, Taylor, McLean, Beard, and Beard (1944 *a*) and Sharp *et al* (1945) have shown that in solutions of sucrose, glycerol, and urea, the sedimentation rates of the influenza virus particles vary appreciably with time and consequently the density values obtained and diameters calculated therefrom may be in error. These authors have studied the sedimentation behavior of three strains of purified influenza virus in bovine albumin which possesses a high molecular weight (70,000) and a low osmotic pressure in the desired density range, and have calculated the densities of the wet virus particles. Their results are shown in Table 3.

TABLE 3

SOME PHYSICAL CONSTANTS OF THREE STRAINS OF INFLUENZA VIRUS

(from Sharp *et al*, 1945)

	A (PR8)	*B (Lee)*	*Swine*
[illegible]	742	840	727
[illegible]	1 104	1,104	1 100
[illegible]	116	124	117
[illegible]	0 822	0 863	0 850
[illegible]	52	34 5	43 3

From the value for the dry density (1/V) and that for wet density, the percentage by volume of water associated with each of the three viruses (hydration) was calculated and is shown in Table 3 Assuming that the virus particles were nearly spherical, a supposition supported by electron micrographs, the diameters were calculated from the wet densities and the sedimentation constants, using a modified form of Stokes' law

$$r^2 = \frac{9\eta s}{2(\sigma - \rho)}.$$

Similar calculations are frequently carried out in virus work, but in general the density used is that of the dried particle since the wet density is seldom known In such a case the calculated diameter is that of the dried particle.

The possibility that some viruses might exist as highly organized cells possessing external envelopes has been considered by Smadel, Pickels, and Shedlovsky (1938) and by Sharp *et al* (1944 *a*, *b*, 1945) Influenza virus particles (PR8 strain) in sucrose solutions rapidly increase in density which is followed after a period by a decrease in density. Such changes were not found in albumin solutions which have a low osmotic pressure despite their high density. According to Sharp *et al.* (1944 *a*, *b*, 1945), if a membrane-like structure surrounding the particles is postulated which permits sucrose to enter but not albumin, the particles would increase in density if placed in a sucrose solution because of loss of water through the membrane. Sucrose would at the same time enter through the membrane, and after an initial period of water loss would reach a sufficient concentration to reverse the flow of water and cause a decrease in density

Opinions contrary to these were expressed earlier by McFarlane, Macfarlane, Amies, and Eagles (1939) who investigated the subject and concluded that the weight of evidence opposed the conception that the virus particle was limited by a semipermeable membrane, and it was more in keeping with the properties of the

vaccinia had no superficial membrane According to Mudd (1944), certain animal viruses and phages showed simple cellular structure, whereas plant viruses appeared to be nucleoproteins devoid of cellular organization Electron microscope studies

mentation constant of $s = 550 \times 10^{-13}$ cm/sec/dyne/gm (550S) with a particle weight of 139×10^{6} (Stern and Duran-Reynals, 1939) Molecular weights of much lower order can also be estimated by centrifugalization (e.g enzymes), and Rothen (1940) was able to estimate the sedimentation velocity of ribonuclease with a molecular weight of 14,000. Lauffer and Stanley (1940) contend that the values elicited by application of the centrifugalization technique are remarkably constant and can readily be repeated at independent laboratories. Beard (1945) states that a population of particles possessing uniform size, density, and shape sediment at a constant rate, and so display evidence of their physical homogeniety in a tissue suspension by the formation of a sharp and smooth boundary descending in the cell during the progress of centrifugalization.

The best effects were observed with rabbit papilloma virus, preparations of which showed sharpness of sedimentation boundary comparable with that of molecular proteins and crystalline plant viruses, indicative of a high state of purity and uniformity of particle size, nevertheless, sedimentation velocity determinations by Neurath *et al.* (1941) on twelve different preparations of highly purified papilloma virus gave results which varied between s = 266 1–287.6 S with an average value of 278 S. *Corrected to 20° C. the average sedimentation constant was* $s_{20} = 280$ S This variation is well outside the error of the method, and it was suggested by Sharp, Taylor, and Beard, (1946) that the variation from one preparation to another might be accounted for on the basis of dissymmetry of shape, or more probably of the amount of water associated with the virus particle.

Equine encephalomyelitis virus produced a less sharp boundary than papilloma virus owing to interference from normal chick embryo tissue component according to Taylor, Sharp, Beard, and Beard (1943), and Sharp, Taylor, Beard, and Beard (1943)

Intensive research by numerous workers has shown that human influenza viruses A and B and the swine virus possess different sedimentation rates, but all show diffuse boundaries indicative of variation in particle size. The behavior of virus concentrated by centrifugalization is identical with that obtained by red cell adsorption and calcium phospate precipitation (Stanley, 1945). Efforts to enhance sharpness of sedimentation boundary by repeated differential centrifugalization were unsuccessful (Sharp, Taylor, McLean, Beard, and Beard, 1944 *a, b,* Lauffer and Stanley, 1944). *Diffuse boundaries suggestive of impurity were also observed in the case of* vaccinia virus (McFarlane *et al*, 1939, Smadel *et al.*, 1940, Beard, Finkelstein, and Wyckoff, 1938). There was evidence to show that in the case of bacteriophage the sedimentation characteristics of a *B. coli* phage named T_2, designated γ by Delbruck and Luria (1942), could be changed by alterations in hydrogen ion concentration. Sharp *et al* (1946) discovered that two sedimentation constants could be demonstrated under different conditions of pH Below pH 5.8 the value for the single sharp boundary was s = about $1{,}000 \times 10^{-13}$ and above this level $s = 700 \times 10^{-13}$ At pH 5 8, both boundaries were seen together No explanation for these findings was apparent although experiments with lucite models sedimenting in salt solutions suggested that the two sedimentation boundaries of bacteriophage might be due to different states of dispersion of the particles, the sharp boundary with $s = 1{,}000 \times 10^{-13}$ might represent the phage particles dispersed as units Yellow fever virus particles likewise sedimented with a blurred boundary estimated at $18\text{–}30 \times 10^{-13}$ according to Pickels and Bauer (1940)

Density Measurements

By altering the density of the fluid in which virus bodies are dispersed, it is possible to change the sedimentation velocity of a virus in the centrifugal field, and hence to determine the density of the particles Thus sucrose, glycerol, and urea have been employed for the study of vaccinia by Smadel, Pickels, and Shedlovsky (1938), and sucrose was used by Lauffer and Stanley (1944) in their study of In-

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Diameter from sed velocity (mμ)	116	124	117
Partial specific volume, V	0 822	0 863	0 830
Per cent water by volume (hydration)	52	34 5	43 3

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on vaccinia virus after peptic digestion have suggested that it contains an outer envelope with a central dense area resembling a nucleus (Dawson and McFarlane, 1948).

Behavior of Elementary Bodies in the Tiselius Electrophoresis Apparatus

Electrically charged particles of colloids and proteins in an electric field behave in a manner similar to that of ions in an electrolyte. Among a mixture of components, particles possessing similar electrical properties migrate at a characteristically uniform rate either to cathode or anode, depending on the particle charge (Tiselius, 1930, 32, 37; Theorell, 1934; Longsworth, 1939). The quantitative electrophoretic analysis of colloids is carried out in a specially designed U-shaped tube of rectangular cross section in which a sharp boundary can be formed between the protein solution and the pure solvent. The migration of this boundary under the influence of an applied electric field is followed by optical methods similar to those used in determining the sedimentation velocity in the ultracentrifuge

Papilloma virus, in a concentration of 2 7 to 4.5 mg per ml, moves in a single sharp boundary in the pH regions 3.78 to 4.1 and 6 54 to 7.7, agglomeration and precipitation occurring about the iso-electric point at pH 5.0 (Sharp, Hebb, Taylor, and Beard, 1942). The infective properties of the virus are stable between the pH limits 3 78 to 7.7. Similar evidence of electrophoretic homogeneity is exhibited by many crystalline proteins and the hemocyanins (Sharp, Cooper, *et al.*, 1942, Abramson, Moyer, and Gorin, 1942, Shedlovsky and Smadel, 1940).

Bourdillon (1940) found the mobility of influenza A virus to be $5\ 5 \times 10^{-5}$ cm^2 $volt^{-1}$. sec^{-1} between pH 7 to 9 (ionic strength = 0 1) and 8×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH 10 (ionic strength 0 04) The rate of migration appeared to be about the same as that of the complement fixing antigen These measurements were not made by an optical method but by estimating the infectivity at different depths in the electrophoresis cell after a given period of electrophoresis. Later more accurate work by Miller, Lauffer, and Stanley (1944) showed that influenza A (PR8) virus purified by fractional centrifugation migrated with a single sharp boundary in the pH regions 2.8–3 0 and 8 8–9 3 The iso-electric point at pH 5.3 was determined by the micro-electrophoresis method of Abramson Similar studies on vaccinia virus have been unsatisfactory according to Beard (1945), the iso-electric point has been stated to lie between pH 4 3 and 4 67 (Beard, Finkelstein and Wyckoff, 1938).

Biochemical Structure of Viruses

The introduction of the electron microscope, the analytical ultracentrifuge and

The chemical composition of several of these purified agents has been investigated, among them vaccinia (Hoagland, 1943), rabbit papilloma (Taylor, Beard, Sharp, and Beard, 1942, Beard, Bryan, and Wyckoff, 1939), equine encephalomyelitis (Taylor, Sharp, Beard, and Beard, 1943), human influenza virus A, B and swine influenza virus (Taylor, 1944).

Results of these analyses are shown in Table 4 and for the sake of comparison analyses are shown for tobacco mosaic virus (Stanley and Loring, 1938) and a normal chick embryo component of high particle weight (Taylor, Sharp, Beard, and Beard, 1942)

The tobacco mosaic virus appears to be composed essentially of protein combined with nucleic acid of the ribose or yeast nucleic type with traces of carbohydrate (Stanley and Loring, 1938) Rabbit papilloma virus appears to be one of the simplest of animal viruses resembling that of plant viruses, and consists of nucleoprotein complex of the desoxypentose type, according to Beard, Bryan, and Wyckoff (1939) The remainder of the animal viruses studied have been found to

TABLE 4

CHEMICAL ANALYSES OF PURIFIED ANIMAL VIRUS PREPARATIONS, THE NORMAL CHICK EMBRYO COMPONENT AND TOBACCO MOSAIC VIRUS
(From Beard, 1945)

(All values are per cent dry weight of the whole virus)

	Whole virus				Lipid				Nonlipid			
	C	N	P	Carbohydrate	Total	Phospholipid	Cholesterol	Neutral fat	Total	Protein	Carbohydrate	Nucleic acid
Vaccinia	33.7	15.3	0.57	2.8	5.7	2.2	1.4	2.2	94	83	2.8	5.0*
Papilloma	49.6	15.0	0.94	6.5	1.5	—	—	—	98.5	90.0	—	8.7*
Equine encephalomyelitis (Eastern strain)	59.0	7.7	1.96	5.2	48.5	30.0	10.0	9.0	54.0	49.0	3.5	4.4†
Influenza A(PR8 strain)	53.2	10.0	0.97	12.5	23.4	11.3	7.0	5.1	77.5	65.0	7.3	1.5*
Influenza B(Lee strain)	52.7	9.7	0.94	13.1	22.4	11.2	3.7	7.2	76.4	63.6	9.42	2.2*
Swine influenza	51.4	9.0	0.87	10.0	24.0	10.7	5.7	7.7	77.6	67.6	10.0	—*
Normal chick embryo component	55.3	0.5	2.3	7.0	35.0	23.4	6.5	6.9	66.5	40.7	9.5	10.6†
Tobacco mosaic	50.7	16.0	0.63	2.5	—	—	—	—	100.0	94.2	2.5	5.8†

* Desoxypentose nucleic acid
† Ribopentose nucleic acid

be more complex and to contain an extra lipid component. Investigations into the composition of vaccinia have shown the nucleic acid to be that of the thymus type with carbohydrate present in amounts normally in excess of that associated with nucleic acid. Lipid in small amounts also forms an integral part of the structure of the elementary bodies of vaccinia and it is also possible that cholesterol is intimately associated with the particles.

The viruses of Eastern and Western encephalomyelitis represent agents of complex character and the variety of nucleic acid recovered from them has been identified as being of the ribose type similar to that of plant viruses. The proportion of lipid material is about 48 per cent. of the virus by weight, the virus can readily be hydrolyzed to produce nucleic acid of high purity. The relatively large quantity of lipid present was sufficient to permit estimation of the phospholipid, cholesterol and neutral fat content (Beard, 1945).

Influenza virus A, B, and the swine influenza virus were also shown to be lipo-nucleoprotein complexes, in which nucleic acid was found to be of the thymus or desoxypentose type. Of special interest was the nitrogen to phosphorous atomic ratio present in the lipid fraction, of the order of two atoms of lipid nitrogen to one of phosphorus, which suggested the existence of diaminomonophospholipid.

Chemical [illegible]
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found to be [illegible]
cholesterol and phospholipid were absent. Desoxypentose and ribopentose nucleic acids were both present, the former in excess of the latter. Comparative observations were made in the bacterial host cell and it was noted that in contrast to the phage, the lipid contained nitrogen and phosphorus and the atomic ratio of the elements indicated a phospholipid of lecithin type. Another feature of difference was the excess of ribopentose nucleic acid over desoxypentose nucleic acid in the bacterial cell.

Purification of Viruses *

Although recent work has shown that several viruses are composed of nucleoprotein, Pirie (1946) points out that nucleoprotein is a normal constituent of macromolecules, and it is not possible to differentiate viruses from normal tissue components by chemical methods. It is equally unwise to presume that all plant viruses are nucleoproteins (Darlington, 1944).

Since the chemical purification of tobacco mosaic virus was accomplished by

* This subject has recently been exhaustively reviewed by Beard (1948).

Stanley (1935–6), and work on similar lines was performed on the cucumber and tomato bushy stunt infections by Bawden and Pirie (1937), a spate of work has ensued on the chemical structure of plant viruses With the aid of the air-driven analytical ultracentrifuge it was possible to separate the infective agent from the sap of infected plants. Chemical studies revealed that the virus of tomato bushy stunt could be isolated in a true crystalline form, and those of tobacco mosaic and cucumber disease in the shape of paracrystals, moreover, they were found to consist of nucleic acid and protein exhibiting the physical behavior of homogeneous macromolecular nucleoproteins (see Wyckoff, Biscoe, and Stanley, 1937; Erikson, Quensel and Svedberg, 1936).

Chemical attempts to purify mammalian viruses have proved to be less successful for obvious reasons The bulk of these agents are intracellular parasites, and are so intimately associated with the complex tissues of the host cell that their separation from such surroundings becomes a formidable task, as Wyckoff (1945) and Beard (1945) have explained. The amounts of virus present in individual tissues vary considerably; for instance, in the case of the rabbit papilloma virus it is necessary to capture a large number of rabbits bearing tumors to provide enough material for extractions. The maximum capacity of the air-driven ultracentrifuge is limited to 200 c.c., but the 50,000 r.p.m. Sharples type of centrifuge may offer greater possibilities for large scale preparation. Viruses such as influenza and equine encephalomyelitis can be cultivated in the allantoic fluid and chicken embryo, and a large bulk of infected material can be gathered. Unfortunately, all living tissues contain much particulate matter, designated normal component, of approximately 25 mμ in size, and thus the separation of equine encephalomyelitis virus particles measuring 40 mμ cannot be accomplished by centrifugalization alone. Prolonged exposure to concentrated electrolytes results in disintegration of the normal component and thereby aids greatly the purification (Taylor, Sharp, Beard, and Beard, 1943). For similar reasons efforts to free the Rous sarcoma virus from chicken tissue component, and poliomyelitis virus from spinal cord have proved troublesome (Claude, 1938, Loring and Schwerdt, 1942).

Owing to the larger particle size of the viruses of vaccinia, influenza, and rabbit papilloma, better results have been obtained Beard (1945) mentions that technical methods other than ultracentrifugation may well be exploited with profit in the purification of viruses There are indications that the relative stability of individual viruses can be affected by variation of pH, temperature, light, salts, and salt concentration Plant viruses can be obtained in an uniform state of dispersion in either dilute buffer solution or water. Vaccinia elementary bodies, according to McFarlane (1940), clump and deposit in normal Ringer and 0 86 per cent. saline solution, but other animal viruses require a minimum concentration of 0 005 M phosphate buffer solution to maintain their respective elementary bodies in uniform dispersal.

Equine encephalomyelitis virus is readily inactivated in normal saline within 24 hours, but is stable for 21 days in Ringer fluid or 0.1 M phosphate buffer (Taylor *et al*, 1944, and Knight, 1944) There are data which also suggest that repeated centrifugation and compression of elementary bodies causes progressive loss of infectivity and irregularity of sedimentation boundary characters (Taylor, Sharp, Beard, and Beard, 1943, Taylor *et al*, 1944)

Partial purification of influenza virus strains PR8, Weiss and Lee, has been accomplished by using ethyl and methyl alcohol under accurately controlled conditions of alcohol-concentration, temperature and pH by Cox, van der Scheer, Aiston and Bohnel (1947). The latter reported that concentration was possible

fected human tissues.

CHAPTER VIII

SOME STATISTICAL METHODS IN VIRUS STUDIES

Estimation of the LD_{50} (Reed and Muench, 1938)

In experiments to ascertain the correlation between the number of elementary bodies and number of infectious units of vaccinia, Parker and Rivers (1936) applied the 50 per cent. end point method described by Reed (1936) and Reed and Muench (1938), developed from the earlier studies of Gaddum (1933) on biological standardization (Wilson and Worcester, 1943) The method of Reed and Muench has since been accorded universal recognition Because of its importance to virus workers we have reproduced the following from their original account, for the convenience of readers in field laboratories with some modifications

In the titration of sera and viruses, the end point is usually taken as that dilution at which a certain proportion of animals react or die Gaddum (1933) drew attention to the advantages of using the dilution at which 50 per cent. of the animals tested are affected, since such an end point is less affected by chance variation than any other, and is infinitely superior to the 100 per cent. end point so frequently employed The ideal method of determining this figure would be to use the largest possible number of animals at dilutions near the value of the 50 per cent reaction, and to interpolate the correct value by the procedures described by Gaddum. Unfortunately, owing to the wide limits and range of titer of biological material, too many animals are required for Gaddum's method. Reed (1936) has conceived an alternative whereby the necessity for using large numbers of animals can be obviated Two critical dilutions between which the end point should lie, are selected and the effect is that of using, at the two critical dilutions, larger groups of animals than are actually included at these dilutions. By inclining to equalize chance variations, the method tends to define the point more nearly than is possible if it is simply interpolated between the two bracketing results Reed and Muench give as an example the following hypothetical result after titration of a protective (neutralizing) serum

TABLE 5

Titration of a Hypothetical Serum
From Reed and Muench (1938)

Dilution	Alive	Dead	Total Alive	Total Dead	Percentage Mortality
a	*b*	*c*	*d*	*e*	*f*
1:1	6	0	32	0	0
1:2	6	0	26	0	0
1:4	5	1	20	1	5
1:8	6	0	15	1	6
1:16	4	2	9	3	25
1:32	2	4	5	7	58
1:64	2	4	3	11	79
1:128	0	6	1	17	94
1:256	1	5	1	22	96

Six mice are used for each dilution, and the number alive and dead entered in columns *b* and *c* The assumption is made that mice alive at one dilution would have survived at a lower serum dilution. Thus column *b* is added from below and the subtotal (i e, 32) entered in column *d* as representing the total number of mice

surviving at this plus higher dilutions. Conversely, it is accepted that mice dead at a given dilution would have died at any higher one, and so column *c* is added from above and the resulting subtotal of 22 entered at the foot of column *e* under dilution 1·256 Column *e*, therefore, represents the number of mice dying at each specified dilution plus lower dilutions The same procedure is followed throughout. From the data obtained in columns *d* and *e*, the percentage mortality is calculated and entered in column *f* Thus, for a total of 16 mice treated which received a 1.8 dilution of serum, 15 survived at that or higher dilutions and one died at 1.8 dilution and lower, the [illegible] Table 5, a graph Fig. 21 [illegible] rvivals The point of inter [illegible] point, and this lies at a serum dilution of approximately 1:30.

The authors of this method point out that the position of the end point would

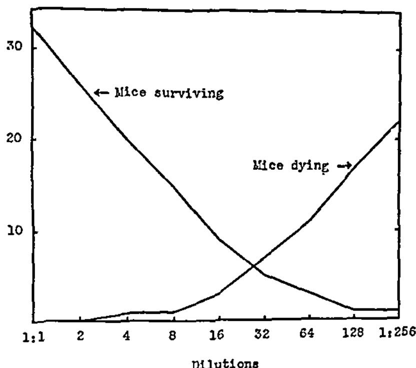


FIG. 21 Graph showing accumulation of deaths and survivals Compare with columns "d" and "e" in Table 5 on page 65 (Reed and Muench)

(Reproduced from *Amer J Hyg*, 1938, 27, 493)

be unaffected by an indefinite number of deaths at higher dilutions or survivals at lower But unfortunately, "accidental" survivals at higher dilutions and deaths at lower would alter the end-point in the direction of the predominant category It is impossible to eliminate individual animals of excessive susceptibility or enhanced resistance, but in spite of obvious limitations, the authors comment that the shift is less for the 50 per cent end point than for any other, and provided that an equal number of dilutions is taken on either side of the end point, errors resulting from accidental deaths should tend to cancel each other For this purpose, an abridged table on the lines of Table 6 is next constructed, with a 1 32 dilution of serum as its central value, because the results indicated that the 50 per cent end point was nearest to this dilution

TABLE 6

ABRIDGED TABULATION OF THE RESULTS OF TABLE 5, BASED ON THE SMALLER NUMBER OF RESULTS (from Reed and Muench, 1938)

Dilution	*Alive*	*Dead*	*Alive*	*Dead*	*Percentage Mortality*
a	*b*	*c*	*d*	*e*	*f*
1:8	6	0	14	0	0
1:16	4	2	8	2	20
1:32	2	4	4	6	60
1:64	2	4	2	10	93
1:128	0	6	0	16	100

(Note: Reed and Muench refer to dilution 1/16 as "the dilution next below," and 1/32 as "the dilution next above" in this particular example.)

The 50 per cent. end-point lies between 1:16 and 1:32, but nearer the latter. It is assumed that since the mortality at 1:32 is 40 per cent. above that at 1:16, while the 50 per cent. end-point is 30 per cent. above, the end-point is 30 per cent./40 per cent., or three-quarters of the distance from 1:16 to 1:32. The formula for the proportionate distance of the end point above the dilution, giving 50 per cent. mortality is:

$$\frac{\text{50 per cent.}-(\text{Mortality at dilution next below})}{(\text{Mortality next above})-(\text{Mortality next below})} = \text{Proportionate distance}$$

The final readings are obtained as follows:

Logarithm of 16 (lower dilution)	1.2041
0.75 (proportionate distance) × log. 2 (dilution factor)	0.2258
Sum (log. of end point)	1.4299

which makes the end point at a dilution of 1:26.9 or approximately 1:27.

The latter part of the calculations can be more rapidly completed by graphic interpolation of the final titer from a sheet of graph paper constructed according to the directions given by Reed and Muench.

This method can be applied to the estimation of the virus-neutralizing properties of any antiviral serum. Should the method be applied to ascertain the infective titer of a virus suspension (instead of, as above, the protective properties of an immune serum), the only alteration required is that the appropriate columns dealing with "deaths" and "survivals" should be reversed in direction. Table 7 shows the result of titrating a hypothetical virus.

TABLE 7

TITRATION OF A HYPOTHETICAL VIRUS

Virus Dilution	*Log. dose*	*Alive*	*Dead*	*Totals Alive*	*Totals Dead*	*Mortality Per cent.*
$\frac{1}{10,000}$	−4.00	0	6	0	16	100
$\frac{1}{32,000}$	−4.50	1	5	1	10	91
$\frac{1}{100,000}$	−5.00	3	3	4	5	55
$\frac{1}{320,000}$	−5.50	4	2	8	2	20
$\frac{1}{1,000,000}$	−6.00	6	0	14	0	0

Calculation of Results.

The 50 per cent. lies between 1/320,000 and 1/100,000 and nearer the latter. The formula for the calculation of the proportionate distance is

$$\frac{\text{50 Per cent} - (\text{Mortality at dilution next below})}{(\text{Mortality at dilution next above}) - (\text{Mortality next below})} = \frac{50 - 55}{20 - 55} = \frac{-5}{-35} = \frac{1}{7} = 0.143$$

Logarithm of the lower dilution	−5.00
Proportionate distance × log dilution factor = .143 × .5 .	.0715
Sum (log of end point)	−5.0715

$$\text{The antilog of } -5.0715 = \frac{1}{117,900}$$

In addition to estimating the infective titer of virus suspensions, this method has been used in more academic studies. Thus it has been used to estimate the degree of infectivity or LD50 of vaccinia virus, and Parker and Rivers (1936) calculated that in the case of one strain (New York Central Laboratory) an average of 42 elementary bodies sufficed to cause skin infection in the rabbit. Employing the time-response relationship method, examination of a different strain of vaccinia studied by Sprunt, Marx, and Beard (1940) showed that the ratio of infectious units to the number of bodies was an average of 1.366. The highest degree of correlation between virus mass and infectious capacity was observed in the case of rabbit papilloma virus, using the incubation period method. Bryan and Beard (1939, 1940) found that the average 50 per cent end point infectious unit or LD50 of the papilloma virus was $10^{-8.855}$ *gm, corresponding to approximately 50,000,000 particles*, indicative of low virulence to the rabbit.

The average 50 per cent. end-points of human influenza A and B virus and the swine virus cultivated in chicken embryo tissues were found to be 80, 600, and 60 *particles, respectively (Beard, 1945). In studies on Eastern equine encephalomyelitis*, Eastern type, it was revealed that 250 particles per 0.05 ml. were required to infect mice (Taylor, Sharp, Beard, and Beard, 1943)

Calculation of the Dosage-Mortality Curve from Small Numbers (Bliss 1938)

Toxicological studies on various multicellular animals show that the typical [illegible] moid type Thus a plot of the [illegible] function of the dosage along [illegible] in percentage kill per unit of the abscissa is smallest near mortalities of 0 and 100 per cent., and largest near 50 per cent.

Bliss (1938) showed that it was possible to convert the sigmoid curve to a straight line by transforming dosage to logarithms, and the percentages to a function of the normal curve, such as the "probit." Persons employing *the method* should read Bliss's paper for the requisite table for conversion of *percentage mortality* to corresponding "probits" (or probability units) for transforming the normal sigmoid dosage curve to a straight line graph—or regression line (see Fig 22 [Probit analysis and statistical treatment of the sigmoid response curve are fully described by Finney (1947)] The method was originally evolved to satisfy the requirements of pharmacologists seeking to measure the toxicity of *varying dilutions* of drugs to small groups of animals, such as for measuring the effect of gelsemicine hydrochloride on New Zealand red rabbits (Chen, Anderson, and Robbins, 1938) It was employed by Richardson and Thurber (1933) to estimate the toxicity of sodium fluoride to grasshoppers The relative merits of typhus vaccines *have also* been assayed by Bliss's method (Fulton *et al*, 1946). The theory and practice of biological assay and a review of 275 publications have been referred to by Bliss and Cattell (1943)

Crawley (1948), who has had practical experience with this method in virus studies has recommended the following procedure when setting up a test Mice do not need to be of uniform age, weight, sex, or homozygous, since the degree of such variation is apparent by the slope of the graphic regression line However, the greater the uniformity of susceptibility among the animals to a particular infection, the more accurate will be the determination of positions on the regression line. The Bliss method has been designed to allow the use of such heterozygous animals as rabbits and grasshoppers, and calculations are based on the underlying principle of the sigmoid character of the dosage-mortality response curve Further-

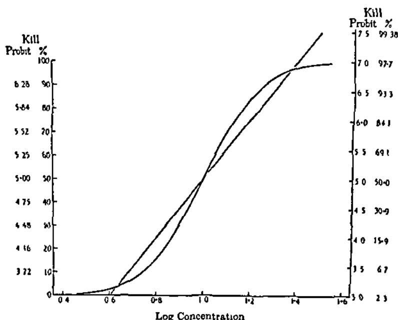


Fig. 22 Bliss's probit method, transformation of sigmoid curve (Reproduced from Finney, D J, 1947, *Probit Analysis*, Cambridge University Press)

more, allowance is made for the number of animals employed and the level of response observed

Owing to the variability of response and the relative degree of suspectibility of the species to various routes of inoculation with different viruses, it is necessary to select that particular route of inoculation which yields the most reliable results For example, when using influenza virus, mice are inoculated intranasally, with the encephalitis group of viruses, the intracerebral route is usually employed, and with the group of viruses which attack the skin the intradermal route is the method of choice.

A two, three, or four dose assay using larger groups of animals is preferable to a multiple dose assay which employs small groups The latter test does not give the maximum obtainable information because of the inevitable wastage of animals at the 0 and 100 per cent levels The doses of virus should be carefully selected so that the graded response may be expected to lie within the range of 5 to 95 per cent., and to "straddle" the 50 per cent end point In order that this range of dosage may be achieved, a carefully titrated stable stock of virus is essential

When planning a test, the number of animals which must be used depends on

the degree of accuracy desired for the material under test. The more accurate the result desired, the greater must be the number.

When performing an antigenicity test on a vaccine, two procedures are possible. Th
live
of
is
vaccine. The second provides a more accurate assessment of the immune response of the animal population to a given stimulation, and approximates more closely to circumstances pertaining to nature.

In preparation for the antigenicity test, the infective titer of the virus to be used for challenge material must be carefully measured. Furthermore, the stock preparation of infective virus must be stable, so that consistent results may be obtained over a long period of time. The LD50 of the virus preparation can be defined within quite narrow limits when the results from the preliminary tests are analyzed statistically. In the case of the stock virus (A) used in the experiments shown in Table 8, the LD50 had been previously calculated to be −6.78 ±0.08.

It is also necessary to know the dosage-response curve for mice vaccinated with the standard vaccine. Trial experiments indicated the LD50 value for mice vaccinated with a given quantity of the standard vaccine (C) was −4.20 ±0.15, and that the average slope of the regression line was 0.80 ±0.20.

Unknown vaccines may be tested by either of the following methods

1. A preliminary trial test is made using small numbers of animals at widely spaced doses, so that the greatest range of activity that might be encountered is covered. In practice this range is narrowed considerably with increasing experience. With this preliminary estimation of the position of the regression line, it is possible to select a dose which will approximate the LD50. The trial test of the *unknown vaccine (B) (Table 8) indicated that the LD50 was approximately −5.60.*

TABLE 8

TABULATED RESULTS OF A HYPOTHETICAL ANTIGENICITY EXPERIMENT IN MICE

Mice	*Log dose*	*Mortalities*	*Probits*	*Calculated LD50*
A				
Unvaccinated controls	−7.60	2/14*	3.93	
(Virus titration)	−6.78	6/15	4.75	−6.65 ± 0.18
	−6.00	12/15	5.84	
B				
Test vaccine treated	−6.80	2/15	3.89	
	−5.60	7/15	4.92	−5.64 ± 0.21
	−4.40	14/15	6.50	
C				
Standard vaccine treated	−5.40	3/15	4.16	
	−4.20	8/15	5.08	−4.34 ± 0.28
	−3.00	14/15	6.11	

* 1 nonspecific death (due to trauma)

2. If numbers of animals and expense are of no concern, then the unknown vaccine may be tested as follows. A sufficient number of groups of mice is vaccinated with the unknown vaccine, so that the entire range of activity of the virus preparation may be covered using evenly spaced doses. In this way the position of the regression line of the unknown vaccine may be obtained with sufficient accuracy so that comparisons can be made with the standard vaccine.

The average slope of the regression lines has been determined by the results of previous experiments. In general, it will be found that vaccinated mice do not respond as uniformly to the inoculation of living virus as do the unvaccinated con-

trols. By the use of the expected slope of the regression lines, it is possible to choose suitable dilution factors for the dosage of virus, so that mortalities between 5 and 95 per cent. may be obtained. The expected LD50 is used as the mid point.

One hundred and thirty-five mice are dealt by random selection into 9 groups of 15 animals each The groups are then assigned to the various positions of the test at random. Three groups are assigned for the purpose of unvaccinated controls (A). Three groups are vaccinated with the test vaccine (B), and the remaining three groups are inoculated with the reference vaccine (C).

After a suitable time interval (usually 14 days) the mice are challenged with known quantities of living virus.

The results of a statistical analysis of the data presented in Table 8 and Fig. 23 show that there is a highly significant (real) difference between the position of

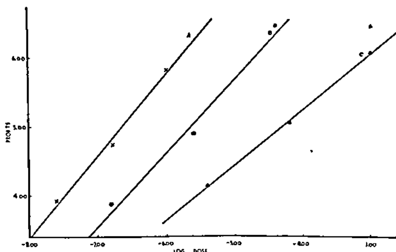


FIG. 23 Bliss's probit method for assay of vaccines, as described in the text

each of the three regression lines This is interpreted as follows The unknown vaccine (B), while giving a significant degree of protection, is not as good as the standard vaccine (C) Since the differences in the results are highly significant, there is no need of further testing of the unknown vaccine (B).

If more exact determinations are required, as in borderline cases, the accuracy of the test may be increased by using larger numbers of animals

It should be noted that the above outline is only a generalized summary of the procedure to follow in an antigenicity test. Each virus has its own particular set of variables, and ways must be devised to meet the particular situation at hand

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CHAPTER IX

THE PARTICULATE NATURE OF VIRUSES—A SUMMARY OF RESULTS

As EARLY AS 1887 Buist of Edinburgh (Gordon, 1937; Mackie and van Rooyen, 1937) and later, Paschen (1906) of Hamburg, observed independently that the causal agent of vaccinia was a minute micro-organism that could be demonstrated in stained preparations under the ordinary microscope. In succeeding years other workers made similar observations in connection with other virus diseases, and at the present time it is generally accepted that the designation "ultramicroscopic and invisible viruses" is frequently a misnomer, for certain are capable of being stained and can be seen with the ordinary microscope. The limit of visibility attainable with the ordinary microscope has been fully discussed elsewhere (Chapter I), and it only remains to repeat that any virus possessing a size of 67 mμ should (according to Coles, 1929) be visible as a stained particle under the ordinary microscope Under darkground illumination the limits of resolution are greatly enhanced, and it should therefore be possible to see particles even smaller than this—a fact which has been fully supported by the observations of Merling-Eisenberg (1938), who succeeded in demonstrating phage particles measuring 25 mμ in size by the use of *darkground illumination and an optical system possessing a numerical aperture of* 1 25.

The following is a list of viruses which have been stained and demonstrated under the microscope in the form of elementary bodies canary-pox, mouse ectromelia, fowl-pox, herpes simplex, inclusion conjunctivitis, infectious myxomatosis of rabbits, Japanese encephalitis, lymphogranuloma inguinale, molluscum contagiosum, measles, psittacosis, pneumonitis, rabbit fibroma of Shope, trachoma, variola, vaccinia, varicella, and zoster. References to literature regarding the elementary bodies found in these diseases are contained in Table 9.

Although these viruses are clearly visible under the light microscope, accurate size measurements are not possible Ultrafiltration and ultracentrifugation have provided evidence that viruses range in size from psittacosis which measures at [illegible] and mouth disease measuring 8–12 mμ The latter [illegible] he molecules of egg albumen (4 mμ), oxyhema- [illegible] (5 mμ), and serum pseudoglobulin (7 mμ), ac- [illegible] lford and Ferry (1934) Since the introduction of the electron microscope, fresh interest has been aroused, and the particulate size of numerous viruses has been reinvestigated The results of the latter method are, in general, in agreement with those of ultrafiltration and ultracentrifugation The electron microscope also may provide information respecting the manner of multiplication of virus particles, and the genesis of inclusions Intensive studies on the origin of bacteriophage particles in parasitized bacterial cells, and related lines of investigation, may answer the keenly debated question whether viruses reproduce like bacteria or by a different mechanism (Hook *et al*, 1946)

TABLE 9—THE SIZE OF VIRUSES

Name of Disease	Size of Virus Estimated by: Ultrafiltration	Centrifugalization	Electron Microscope (or ultraviolet light)	Authority	Elementary Bodies Demonstrated in Stained Film Preparations under Ordinary Microscope by
Aujeszky's disease ("Mad itch," pseudorabies or infectious bulbar paralysis)	100–150	—	—	Elford and Galloway (1936)	—
Avian encephalomyelitis	20–30	—	—	Olitsky and Bauer (1939)	—
Baker's feline pneumonitis	—	—	466 ± 788	Kurotchkin *et al* (1947)	
Borna disease of horses	85–125	—	—	Elford and Galloway (1933)	
Bwamba fever virus	113–150	—	—	Smithburn, Mahaffey, and Paul (1941)	
Canary-pox	125–175	—	(125–175 by UVL)	Burnet and Barnard (1933)	Herzberg (1936)
Colorado tick fever virus	24	—	—	Florio, Stewart, and Mugrage (1946)	
Durand's virus	53–80	—	—	Findlay (1942)	
Ectromelia	100–150	—	(130–140 by UVL)	Barnard and Elford (1931)	Marchal (1930), Paschen (1936)
Ectromelia	—	—	300 by 210	Boswell (1947)	
Equine encephalomyelitis	20–30	—	—	Bauer, Cox, and Olitsky (1935)	
Equine encephalomyelitis	20–35	32–39	—	Tang, Elford and Galloway (1937)	
Equine encephalomyelitis (Eastern strain)	—	—	42 0	Taylor, Sharp, Beard, and Beard (1942)	
Equine encephalomyelitis (Western strain)	—	—	39 8	Beard (1945), Sharp, Taylor, Beard, and Beard (1943)	
Foot and mouth disease	8–12	—	—	Galloway and Elford (1931); Krassnoff and Reinié (1937)	
Foot and mouth disease	—	17–20	—	Elford and Galloway (1937)	
Fowl plague	60–90	—	—	Elford and Todd (1933), Burnet and Ferry (1934)	
Fowl-pox	—	—	322 by 264	Boswell (1947)	Borrel (1904); Woodruff and Goodpasture (1929, 1930), Ledingham (1931)
Herpes simplex	100–150	—	—	Elford, Perdrau, and Smith (1933), Païc, Krassnoff and Reinié (1938)	Taniguchi *et al* (1934); Amies (1934), Herzberg (1936)
Inclusion conjunctivitis	231	—	—	Thygeson (1934*a*), Tilden and	Lindner (1909, 1910,

CHAPTER IX

THE PARTICULATE NATURE OF VIRUSES—A SUMMARY OF RESULTS

As EARLY AS 1887 Buist of Edinburgh (*Gordon, 1937, Mackie and van Rooyen, 1937*) and later, Paschen (1906) of Hamburg, observed independently that the causal agent of vaccinia was a minute micro-organism that could be demonstrated in stained preparations under the ordinary microscope. In succeeding years other workers made similar observations in connection with other virus diseases, and at the present time it is generally accepted that the designation "ultramicroscopic and invisible viruses" is frequently a misnomer, for certain are capable of being stained and can be seen with the ordinary microscope. The limit of visibility attainable with the ordinary microscope has been fully discussed elsewhere (Chapter I), and it only remains to repeat that any virus possessing a size of 67 mμ should (according to Coles, 1929) be visible as a stained particle under the ordinary microscope Under darkground illumination the limits of resolution are greatly enhanced, and it should therefore be possible to see particles even smaller than this—a fact which has been fully supported by the observations of Merling-Eisenberg (1938), who succeeded in demonstrating phage particles measuring 25 mμ in size by the use of darkground illumination and an optical system possessing a numerical aperture of 1 25.

The following is a list of viruses which have been stained and demonstrated under the microscope in the form of elementary bodies canary-pox, mouse ectromelia, fowl-pox, herpes simplex, inclusion conjunctivitis, infectious myxomatosis of rabbits, Japanese encephalitis, lymphogranuloma inguinale, molluscum contagiosum, measles, psittacosis, pneumonitis, rabbit fibroma of Shope, trachoma, variola, vaccinia, varicella, and zoster. References to literature regarding the elementary bodies found in these diseases are contained in Table 9

Although these viruses are clearly visible under the light microscope, accurate size measurements are not possible Ultrafiltration and ultracentrifugation have provided evidence that viruses range in size from psittacosis which measures at least 230 mμ to the agent of foot and mouth disease measuring 8–12 mμ. The latter approximates in dimensions to the molecules of egg albumen (4 mμ), oxyhemaglobin (4 5 mμ), serum albumin (5 mμ), and serum pseudoglobulin (7 mμ), according to Elford (1933) and Elford and Ferry (1934) Since the introduction of the electron microscope, fresh interest has been aroused, and the particulate size of numerous viruses has been reinvestigated The results of the latter method are, in general, in agreement with those of ultrafiltration and ultracentrifugation The electron microscope also may provide information respecting the manner of multiplication of virus particles, and the genesis of inclusions Intensive studies on the origin of bacteriophage particles in parasitized bacterial cells, and related lines of investigation, may answer the keenly debated question whether viruses reproduce like bacteria or by a different mechanism (Hook *et al*, 1946)

TABLE 9—THE SIZE OF VIRUSES

Name of Disease	*Size of Virus Estimated by:* *Ultrafiltration*	*Centrifugalization*	*Electron Microscope (or ultraviolet light)*	*Authority*	*Elementary Bodies Demonstrated in Stained Film Preparations under Ordinary Microscope by*
Aujeszky's disease ("Mad itch," pseudorabies or infectious bulbar paralysis)	100–150	—	—	Elford and Galloway (1936)	—
Avian encephalomyelitis	20–30	—	—	Olitsky and Bauer (1939)	—
Baker's feline pneumonitis	—	—	466 ± 788	Kurotchkin *et al.* (1947)	
Borna disease of horses	85–125	—	—	Elford and Galloway (1933)	
Bwamba fever virus	113–150	—	—	Smithburn, Mahaffey, and Paul (1941)	
Canary-pox	125–175	—	(125–175 by UVL)	Burnet and Barnard (1933)	Herzberg (1936)
Colorado tick fever virus	24	—	—	Florio, Stewart, and Mugrage (1946)	
Durand's virus	53–80	—	—	Findlay (1942)	
Ectromelia	100–150	—	(130–140 by UVL)	Barnard and Elford (1931)	Marchal (1930), Paschen (1936)
Ectromelia	—	—	300 by 210	Boswell (1947)	
Equine encephalomyelitis	20–30	—	—	Bauer, Cox, and Olitsky (1935)	
Equine encephalomyelitis	20–35	32–39	—	Tang, Elford and Galloway (1937)	
Equine encephalomyelitis (Eastern strain)	—	—	42.0	Taylor, Sharp, Beard, and Beard (1942)	
Equine encephalomyelitis (Western strain)	—	—	39.8	Beard (1945), Sharp, Taylor, Beard, and Beard (1943)	
Foot and mouth disease	8–12	—	—	Galloway and Elford (1931), Krassnoff and Reinié (1937)	
Foot and mouth disease	—	17–20	—	Elford and Galloway (1937)	
Fowl plague	60–90	—	—	Elford and Todd (1933), Burnet and Ferry (1934)	
Fowl-pox	—	—	322 by 264	Boswell (1947)	Borrel (1904), Woodruff and Goodpasture (1929, 1930), Ledingham (1931)
Herpes simplex	100–150	—	—	Elford, Perdrau, and Smith (1933), Païc, Krassnoff and Reinié (1938)	Taniguchi *et al.* (1934), Amies (1934); Herzberg (1936)
Inclusion conjunctivitis	233	—	—	Thygeson (1934*a*), Tilden and	Lindner (1909, 1910,

TABLE 9—*Continued*—THE SIZE OF VIRUSES

Name of Disease	*Size of Virus Estimated by* Ultrafiltration	Centrifugalization	Electron Microscope (or ultraviolet light)	*Authority*	*Elementary Bodies Demonstrated in Stained Film Preparations under Ordinary Microscope by*
Inclusion conjunctivitis	153–206	—	—	Gifford (1936), Julianelle, Harrison and Lange (1938)	1911), Thygeson and Mengert (1936)
Inclusion conjunctivitis	200	—	—		
Infectious myxomatosis of the Rabbit (Sanarelli)	—	—	—		Aragão (1911), van Rooyen (1937)
Influenza swine virus	—	81.5	—	Beard *et al* (1944)	
Influenza swine virus	—	—	77.6	Beard *et al* (1944)	
Influenza swine virus	80–120	—	—	Elford, Andrewes, and Tang (1936)	
Influenza swine virus	—	117	96.5	Sharp, Taylor, McLean, Beard, and Beard (1945)	
Influenza A human (strain PR8)	—	116	101	Sharp, Taylor, McLean, Beard, and Beard (1945)	
Influenza A, human strain (PR8)	—	70	100	Stanley (1944, 1946)	
Influenza B, human strain (Lee)	—	124	123	Sharp, Taylor, McLean, Beard, and Beard (1945)	
Influenza A, human strain (WS)	—	87–99	—	Elford and Andrewes (1936)	
Japanese encephalitis	—	—	—	—	Taniguchi *et al* (1935)
Lymphogranuloma inguinale	125–175	—	—	Miyagawa *et al* (1935)	Miyagawa *et al* (1935), Findlay, Mackenzie, and MacCallum (1938)
Lymphogranuloma inguinale	125–175	—	—	Broom and Findlay (1936)	
Lymphogranuloma inguinale	100–140	—	—	Levaditi, Païc, and Krassnoff (1936*b*)	
Lymphogranuloma inguinale	—	—	438 ± 47	Kurotchkin *et al* (1947)	
Louping-ill	15–20	—	—	Elford and Galloway (1933*a*)	
Louping-ill	—	22–27	—	Tang, Elford, and Galloway (1937)	
Lymphocytic choriomeningitis	100–150	—	—	Rivers and Scott (1936)	
Lymphocytic choriomeningitis	33–50	—	—	Casals-Ariet and Webster (1940)	
Meningopneumonitis	—	—	354 ± 41	Kurotchkin *et al* (1947)	
SF pneumonitis	—	—	422 ± 58	Kurotchkin *et al* (1947)	
Molluscum contagiosum	—	—	302 by 226	Boswell (1947)	Lipschutz (1911); von Prowazek (1911), van Rooyen (1938)
Morbilli	—	—	—		Taniguchi *et al* (1935*a*)
Newcastle disease of fowls	80–120	—	—	Burnet and Ferry (1934)	
Nigg's mouse pneumonia virus	—	—	497 ± 77	Kurotchkin *et al* (1947)	
Pneumonia virus of mice (PVM)	100–150	—	—	Horsfall and Hahn (1940)	

TABLE 9—*Continued*—THE SIZE OF VIRUSES

Name of Disease	*Size of Virus Estimated by* *Ultrafiltration*	*Centrifugalization*	*Electron Microscope (or ultraviolet light)*	*Authority*	*Elementary Bodies Demonstrated in Stained Film Preparations under Ordinary Microscope by:*
Poliomyelitis	12–17	—	—	Theiler and Bauer (1934)	
Poliomyelitis	8–12	—	—	Elford, Galloway, and Perdrau (1935), Kling *et al* (1938)	
Poliomyelitis (SK strain)	10–15	—	—	Sanders and Jungeblut (1942)	
Poliomyelitis (SK strain)	—	—	25–30	Jungeblut and Bourdillon (1943)	
Poliomyelitis (Lansing)	—	—	12–34	Loring, Marton, and Schwerdt (1946)	
Psittacosis	220–230	—	—	Levinthal (1935)	Coles (1930); Lillie (1930), Levinthal (1930).
Psittacosis	—	—	455 ± 78	Kurotchkin *et al* (1947)	
Ornithosis	—	—	422 ± 68	Kurotchkin *et al* (1947)	
Rabies (fixed virus)	100–150	—	—	Galloway and Elford (1936), Yaoi, Kanazawa, and Sato (1936)	
Rabies (street virus)	160–240	—	—	Païc, Krassnoff, Haber, Reinié, and Voet (1938)	
Rabbit fibroma virus of Shope	125–175	126–141	—	Schlesinger and Andrewes (1937)	Paschen (1937)
Rabbit papilloma virus of Shope	23–35	32–50	—	Schlesinger and Andrewes (1937)	
Rabbit papilloma virus of Shope	—	—	44	Beard, Bryan, and Wyckoff (1939), Neurath *et al* (1941)	
Rift Valley fever	23–35	—	—	Broom and Findlay (1933)	
Rous sarcoma	75–100	—	—	Elford and Andrewes (1935); Yaoi and Nakahara (1935)	
Rous sarcoma	—	60–70	—	Elford and Andrewes (1936)	
Rous sarcoma	—	70	—	Stern and Duran-Reynals (1939)	
St Louis encephalitis	22–33	—	—	Bauer, Fite, and Webster (1934)	
St Louis encephalitis	20–30	—	—	Elford and Perdrau (1935)	
Sandfly fever	160	—	—	Shortt, Pandit, and Rao (1938)	
Theiler's mouse poliomyelitis	9–13	—	—	Theiler and Gard (1940)	
Theiler's mouse poliomyelitis	—	—	15	Gard (1943)	
Trachoma	200	—	—	Thygeson (1934); Thygeson and Proctor (1935)	Halberstaedter and von Prowazek (1907); Halberstaedter (1912)

TABLE 9—*Continued*—THE SIZE OF VIRUSES

Name of Disease	*Size of Virus Estimated by* Ultrafiltration	Centrifugalization	Electron Microscope (or ultraviolet light)	*Authority*	*Elementary Bodies Demonstrated in Stained Film Preparations under Ordinary Microscope by:*
Vaccinia	125–175	—	—	Elford and Andrewes (1932)	Buist (1887; see Gordon, 1937; Mackie and van Rooyen, 1937), Paschen (1906, 1913), Ledingham (1931); Nauck and Paschen (1933), Smadel, Rivers, and Pickels (1939)
Vaccinia	140–160	—	—	Levaditi, Paic, and Krassnoff (1936*a*)	
Vaccinia	—	170–180	—	Elford and Andrewes (1936)	
Vaccinia	—	103–240	—	McIntosh and Selbie (1937)	
Vaccinia	—	236–252	—	Smadel, Pickels, and Shedlovsky (1938)	
Varicella	—	—	145	Ruska (1943)	Aragao (1911*a*); Paschen (1917, 1933)
Varicella	—	—	210–238	Nagler and Rake (1948)	
Variola	—	—	244–302	Nagler and Rake (1948)	van Rooyen (d) Illingsworth (1944)
Vesicular stomatitis	70–100	—	—	Galloway and Elford (1933), Bauer and Cox (1935)	
Vesicular stomatitis	60–90	—	—	Levaditi, Paic, Krassnoff, and Voet (1936)	
Vesicular stomatitis	—	60–74	—	Elford and Galloway (1937)	
West Nile fever virus	21–31	—	—	Smithburn *et al* (1940)	
Yellow fever	18–28	—	—	Findlay and Broom (1933)	
Yellow fever	15–22	—	—	Bauer and Hughes (1934)	
Yellow fever	—	12–19	—	Pickels and Bauer (1940)	
Zoster	—	—	—		Paschen (1933); Taniguchi *et al.* (1934); Amies (1934)

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CHAPTER X

INCLUSION BODIES

The majority of virus diseases is characterized by the occurrence of organized structures in the cytoplasm or the nucleus of infected tissue cells. These structures are known as inclusion bodies, inclusions, or virus inclusions, it is necessary to define what is meant by these terms. Histologists sometimes use the term "inclusion body" in a broad sense, to refer to any structure in the cytoplasm of a cell other than such purely normal constituents as the mitochondria, Golgi apparatus, or centrosome; thus, they might refer to globules of fat or particles of glycogen as inclusion bodies. Virus workers use the term in a restricted sense, to refer to peculiar intracellular structures generally regarded as characteristic of virus infections. These inclusions may occur in the nucleus or cytoplasm, and be eosinophilic or basophilic. The question of terminology is somewhat complicated, however, by the fact that bodies similar to those occurring in virus infections may occur intracellularly in tissues which are either healthy, or else affected by a disease of nonvirus etiology. For example, while one talks about the Negri body as the characteristic inclusion body of rabies, one also says that inclusion bodies have been found in whooping-cough and brain tumors, both probably nonvirus conditions. We find *the term "virus inclusion" a useful one, but its use does not imply that we necessarily* believe all such structures to be composed solely of virus particles.

In this chapter we intend to discuss the inclusions of human viruses, and the better known inclusions of animal viruses, inclusions have also been reported in various diseases of plants, fish, and insects (Rivers, 1928, Ludford, 1930, Smith, 1930, St John-Brooks, 1930).

Concerning literature, a number of valuable papers of a general nature has appeared in recent years, some giving illustrations (Findlay and Ludford, 1926, Rivers, 1928, Ludford, 1930, Cowdry, 1934, Ishimitsu, 1937).

Classification. Various types of classification may be used in describing these structures. For example, Lipschutz (1921) divided inclusions into three groups. the cyto-oikon or intracytoplasmic body; the karyo-oikon or intranuclear body, and the cyto-karyo-oikon, the body that occurs in both sites. This terminology, however, is scarcely used at the present it is so much easier to refer simply to intracytoplasmic or intranuclear inclusions.

Cowdry (1934) has published a valuable classification of intranuclear bodies, which are divided into Types A and B, according to morphology

THE MORPHOLOGY OF INCLUSION BODIES

Intracytoplasmic

(*a*) *Large eosinophilic inclusions* measure from about 5 μ up to 20 μ in diameter. They are usually spherical, but may be oval, elongated, or triangular. These bodies commonly appear more or less homogeneous, but special methods (*vide infra*) may detect granularity. The better known virus inclusions of this type are the following the Guarnieri body of variola-vaccinia, the Negri body of rabies, the Henderson-Paterson body of molluscum contagiosum, the Bollinger body of fowl-pox, and the Marchal body of mouse ectromelia

(*b*) *Small eosinophilic inclusions* These inclusions are usually early forms of [illegible] to form a larger struc-

[illegible] ia.

[illegible] ually formed by a col-

lection, of a colony, [illegible] The typical appearance

is usually of discrete blue granules lying in a more or less circumscribed area of the cytoplasm. Small basophilic inclusions occur in encephalitis lethargica, and herpes febrilis

(*d*) *Large basophilic inclusions* Large basophilic homogeneous inclusions have been described in lymphogranuloma inguinale (Gamna-Favre bodies). Studies have shown, however, that they are probably nuclear in origin, and not true virus inclusions

(*e*) *The trachoma type of inclusion* The inclusion bodies of trachoma, inclusion conjunctivitis, psittacosis, LGI, and pneumonitis have so many features in common that they can be described together. Thus, with certain minor differences, they each undergo a series of distinctive changes while in process of development, they stain readily with Giemsa's solution, and during the early stages of growth they appear as basophilic intracytoplasmic structures As time proceeds, the inclusions not only increase in size and alter in shape, but also exhibit a tendency to acidophilic characters The mature inclusion body consists of a clump of pink-stained elementary bodies which are sometimes covered by a film of cell material stained light blue, so that the structure as a whole has a lilac tint. This appearance is most marked in the inclusion bodies of trachoma and those of inclusion blennorrhea, and is less obvious in the case of the psittacosis group of agents

During the last decade the inclusions of psittacosis have been exhaustively investigated by Bedson and Bland (see Chapter LXXI), who showed that the earliest visible growth phase of the parasite took the form of a large, apparently homogeneous, basophilic and intracytoplasmic structure which they called the plaque or plasmodium stage. As growth progressed the plaque grew in size and underwent segmentation, so that it eventually assumed the appearance of a segmented mass, called a morula Owing to the resemblance of these initial phases to the early forms of a protozoal bird parasite, Bedson expressed the view that the psittacosis virus showed morphologic similarities more related to the protozoa than to the bacteria Subsequent work by Bedson and Bland however, showed that the earliest developmental forms were in reality composed of an aggregation of smaller masses As the inclusions matured, minute pink-staining elementary bodies began to develop within them and eventually these filled the whole cell, which subsequently ruptured and discharged its contents into the surrounding tissue spaces. The free-lying virus bodies next entered healthy endothelial cells and commenced a second cycle.

The inclusion bodies of psittacosis, trachoma, and inclusion conjunctivitis constitute a unique group of structures. Owing to the facility with which they can be demonstrated in tissues and the color effects which are obtainable in film preparations, they have been examined by cytologists, certain of whom have suggested that the appearances of the inclusion body at different stages of its growth are not solely produced by the parasite itself but are also contributed to by the cell Levinthal (see Chapter LXXI), for instance, goes so far as to assert that the initial forms referred to as the plaque, plasmodium, and morula varieties represent, to a large extent, the reaction of the parasitized cell and, to a lesser degree, the virus itself. This opinion is somewhat similar to that expressed in the past by early German writers in respect of the nature of the trachoma inclusion body. They, for example, pointed out that the light blue-staining masses which developed in the cytoplasm of invaded epithelial cells after infection represented a product of cell degeneration, on account of which they called them "plastin masses" The successive developmental stages of the trachoma inclusion body have been investigated in great detail by Halberstaedter and Prowazek (see Chapter LXV), and from their survey of the literature on this subject they concluded that the first sign of growth of the virus was the formation of the initial body, inside which in turn elementary bodies, or *Strongyloplasma* as they were called at the time, began to appear This process started in the initial bodies situated at the center of the cytoplasm and gradually involved those situated peripherally, so that eventually the whole cytoplasm be-

came filled to bursting point with elementary bodies, the nucleus became pushed to one side, and the cell ruptured.

Much of what has been said regarding the morphology of the Halberstaedter-Prowazek inclusion body is equally applicable to the inclusion body of inclusion blennorrhea, and for all practical purposes they may be regarded as identical in appearance. The only outstanding difference between the two inclusions lies in their relative distribution in affected tissues, for whereas in trachoma the bodies occur only in the conjunctival epithelium, the virus of blennorrhea has also been demonstrated in the mucous membrane of the cervix uteri and, on rare occasions, of the male urethra.

An interesting observation which has been made regarding this group of inclusions is the fact that the elementary bodies appearing in the fully developed trachoma inclusion body are embedded in a jelly-like material; this substance sometimes clings to the elementary bodies after they have been liberated It is this mucoid covering which produces the halo-like zone of nonstaining material that surrounds individual elementary bodies in Giemsa-stained films (see p. 698).

The researches of Bland and Canti (see p 119), who obtained a cinephotomicrographic record of the growth phases of psittacosis virus in tissue cultures, also revealed that the inclusion body possessed a definite outer covering or envelope composed of a relatively dense substance (see p. 750). Kurotchkin *et al.*, (1947) find that treatment of yolk sac smears with ether results in the disappearance of the large forms of LGI, psittacosis pneumonitis viruses, the EB's being unaffected

The microchemical reactions of the inclusion body of trachoma have been investigated by Rice, who found that the matrix consisted of a carbohydrate substance which yielded a specific color reaction with iodine, furthermore, the inclusion could be deprived of this property by preliminary exposure to saliva. The inclusion body of inclusion conjunctivitis has also been found to react in a similar manner.

Findlay and his co-workers (1938) claim that LGI virus also undergoes a developmental process similar to that of psittacosis (see p. 774)

Intranuclear

Cowdry (1934) states that the characteristic features of these inclusions are as follows eosinophilic staining reaction, presence of an unstained halo between the inclusion and the nuclear membrane, and margination of basichromatin on the nuclear membrane Cowdry also reports that intranuclear inclusions can be divided into Types A and B

Type A inclusions are either amorphous, granular, or occur in rounded masses The nuclear material is severely disorganized, and the basichromatin collects on the nuclear membrane These inclusions contain no iron or thymonucleic acid, and micro-incineration leaves no ash. Affected tissues show bodies in all stages of development, thus, certain cells may contain fully developed inclusions, while nearby, only very small forms may occur There is usually a severe tissue reaction in the neighborhood of Type A bodies Type A inclusions are found most characteristically in herpes febrilis, yellow fever, chickenpox, zoster, and virus III infection of rabbits

Type B inclusions cause much less reaction in the nucleus than do Type A The inclusions themselves are more circumscribed, they vary in size and there may be a number in any one nucleus, there is no margination of basichromatin, and there is no marked tissue reaction in the surrounding areas Type B inclusions occur, for example, in poliomyelitis, Rift Valley fever, and Borna disease of horses

VIRUS DISEASES OF MAN IN WHICH INCLUSION BODIES OCCUR

Intracytoplasmic

Eosinophilic. Encephalitis lethargica (Dawson's bodies), Japanese encephalitis (Taniguchi's bodies); molluscum contagiosum (Henderson-Paterson bodies);

Intranuclear

Type A Herpes febrilis (Lipschütz bodies), herpes zoster (Lipschütz bodies); varicella, virus "B" infection, yellow fever. Intranuclear inclusions resembling those of herpes have been described in cases of subacute "inclusion" encephalitis occurring in children, the bodies are found in the neurones in the cortex, basal ganglia, inferior olive, and the horns of the spinal cord, they may also be found in the oligodendroglia (Brain, 1942–3, Greenfield, 1942–3, Russell, 1942–3, Baar, 1946).

Type B. Herpes febrilis (Nicolau's bodies), herpes zoster (Nicolau's bodies); poliomyelitis, Rift Valley fever.

CELL INCLUSIONS FOUND IN HUMAN TISSUES IN NONVIRUS DISEASES

Inclusions have been described in certain conditions for which no virus is held responsible. Thus intranuclear inclusions have been found in nerve cells of persons dying from different diseases (Wolf and Orton, 1932). Nuclear inclusions were found in the tubular epithelium of the kidney and in liver cells in a number of cases of lead poisoning in children (Blackman, 1936). A number of authors have described Type A intranuclear inclusions in the lungs of children dying from whooping-cough pneumonia (Rich, 1932, Kuttner and Wang, 1934, McCordock and Smith, 1934). Goodpasture *et al* (1939) described inclusions in the nuclei of epithelial cells in the respiratory tract of infants after measles or whooping-cough

Broadhurst *et al* (1939) found cytoplasmic inclusions in the respiratory system and blood cells after scarlet fever In later papers they refer to finding stippled bodies in the epithelium of the throat in persons complaining of "irritation" (Broadhurst, Maclean, and Taylor, 1943 *a*, *b*) Cytoplasmic inclusions may occur in the lymph nodes in Hodgkin's disease, and have been studied in tissue culture (Grand, 1944). Eosinophilic cytoplasmic inclusions have been described in tropical phlebitis (Fisher, Fisher, and Lendrum, 1947)

Gliomata, mainly those of the spongioblastoma multiforme type, may contain large eosinophilic intracytoplasmic inclusions (Russell, 1932, Wolf and Orton, 1933–4) Similar bodies may occur in meningioma, perineural fibroblastoma, and hemangioblastoma (Wolf and Orton, 1933–4) Bland and Russell (1938) described intranuclear inclusions in cultures of meningiomata

INTRACELLULAR INCLUSIONS FOUND IN APPARENTLY HEALTHY HUMAN TISSUES

Structures resembling virus inclusions have been found in the tissues of healthy persons on a number of occasions

Type A intranuclear inclusions were found in the intestines, liver, and lungs of a man aged 36 (VonGlahn and Pappenheimer, 1925)

Type B inclusions have been found in the kidneys in a number of healthy human beings (Cowdry *et al*, 1935), and in the male genital tract in the columnar cells of the part derived from the wolffian duct, being closely associated with spermatogenesis (Gilmour, 1937)

A number of authors has described intranuclear inclusions in healthy young children (e.g., Goodpasture and Talbot, 1921; Farber and Wolbach, 1932; Kuttner and Wang, 1934; Pappenheimer and Hawthorne, 1936, for other references see VonGlahn and Pappenheimer, 1925) The patients were usually infants; sometimes still-born fetuses were examined. The inclusions occurred in the bile ducts, intestine, kidneys, liver, lungs, pancreas, parotids, thyroid, and respiratory tract Farber and Wolbach found both [illegible]
of submaxillary glands examined
by Kuttner and Wang (1934) F
of infants are Cappell and McFarlane (1947) and Kalfayan (1947).

Acidophilic intranuclear inclusions appeared spontaneously in cultures of human fetal leptomeninges, and in fibroblast cultures from human fetal lung (Fischmann and Russell, 1940).

CELL INCLUSIONS OBSERVED IN ANIMAL VIRUS DISEASES

Intracytoplasmic

Eosinophilic. Canary-pox; dog distemper, ectromelia (Marchal bodies); foot-and-mouth disease in animals, fowl-pox (Bollinger bodies); louping-ill; myxomatosis of rabbits, ferret epizootics (Slanetz and Smetana, 1937, Spooner, 1938); fowl plague, sheep-pox, cow-pox.

Basophilic. Psittacosis, ornithosis, and pneumonitis.

Intranuclear [1]

Type A. Fox encephalitis, louping-ill, infectious tracheitis of chickens, an owl disease, parrot and parakeet disease, pseudorabies, rats infected with an active principle in sewage (Hindle and Stevenson, 1929–30, Hindle, 1932), salivary gland virus of guinea-pigs, generalized visceral disease of guinea-pigs (Pappenheimer and Slanetz, 1942), salivary gland virus of mice; salivary gland virus of rats, virus III of rabbits, virus of feline agranulocytosis (Hammon and Enders, 1939, Syverton *et al*, 1943).

Type B. Borna disease of horses, infection of the livers of mice (Findlay, 1932).

CELL INCLUSIONS OCCURRING IN HEALTHY ANIMAL TISSUES

Inclusions have been found in a number of apparently healthy animals, it should be realized, however, that viruses may be present in these conditions as it is now known that there exists a number of more or less benign virus infections of animals

Monkeys. Intranuclear inclusions have been found in the respiratory system and bile ducts (Covell, 1932), in the kidneys (Cowdry and Scott, 1935 *b*), and in the liver cells [illegible] .6)

Dogs. [illegible] demonstrated in the liver cells of 2 dogs by [illegible] inclusions were demonstrated in the liver and kidney cells of dogs over two years of age (Nicolau and Kopciowska, 1936).

Cats Normal cats may show small intracytoplasmic granules in their nerve cells (Negri-Luzzani, 1905, 1913)

Guinea-pigs Pappenheimer and Hawthorne (1936) found nuclear inclusions in the liver cells

Mice Negri-like bodies may occur in the nerve cells (Nicolau, Kopciowska, and Balmus, 1933) or liver (Campbell, 1939)

Rats Hurst *et al*, (1943) found intranuclear inclusions in the kidneys of a number of wild rats (see also Syverton and Larson, 1947)

Opossums Hurst *et al*, (1943) found intranuclear inclusions to develop in the kidneys of Australian opossums kept under hygienic laboratory conditions

[1] Many of these inclusions have been personally studied by Cowdry (1934)

Ferrets. Inclusion bodies were found in the nuclei of the liver cells by Pappenheimer and Hawthorne (1936).

Moles. Intranuclear bodies were found in the salivary glands by Rector and Rector (1934).

Birds. Type A lesions were found in the kidneys of a Guatemalan amazon, Type B lesions were found in the lungs of this species and the kidneys of a number of other birds (Cowdry *et al.*, 1935).

Frogs. Cowdry (1934) found Type A lesions in the kidneys of frogs.

Tissue cultures. Acidophilic intranuclear inclusions have been described in cultures of leptomeninges from chick and rat embroys, and in fibroblast cultures from the lungs of rat fetuses (Fischmann and Russell, 1940)

MULTIPLE INCLUSIONS

Under certain conditions, single cells may show more than one type of inclusion Thus, Syverton and Berry (1947 *a*) found that the cells of a Shope's rabbit papilloma could be injected with B virus and myxoma They also showed that the cells of the rabbit cornea might show cytoplasmic vaccinial inclusions, and nuclear inclusions of B virus or herpes (1947 *b*).

THE PRODUCTION OF INCLUSIONS BY PHYSICAL AND CHEMICAL AGENTS

A number of physical and chemical factors have been said to cause inclusions when inoculated into experimental animals.

Intracytoplasmic. 1. Russell's viper venom produced Negri-like bodies in nerve cells in guinea-pigs (Acton and Harvey, 1911).

2. *B. pyocyaneus* injections produced Negri-like bodies in rabbits (Acton and Harvey, 1911).

3. Prolonged administration of pilocarpine produced Negri-like bodies in the salivary glands (Shortt and Lahiri, 1934).

Intranuclear. 1. Inclusions were produced in guinea-pigs by injections of certain aluminium, carbon, and iron compounds, these structures gave negative Feulgen and MacCallum reactions, and so were clearly not of nuclear origin (Olitsky and Harford, 1937).

2. Bismuth injections gave rise to nuclear inclusions in rats (Pappenheimer and Maechling, 1934)

3 Lee (1933–4) produced intranuclear inclusions in the nerve cells of cats after intravenous injection of glucose, common salt, sodium bicarbonate, and distilled water After injection of salyrgan, he found inclusions in the pancreas, kidneys, testes, and suprarenals In a more detailed study (1936) he produced Type B lesions in the cells of the anterior horn by intravenous injection of distilled water Following injection of hypertonic glucose, glucose plus acacia, common salt, and sodium bicarbonate, Type A lesions developed in Purkinje cells, in anterior horn cells, in cells of the spinal and sympathetic ganglia, and in pyramidal cells

4 Nuclear inclusions were produced in the kidneys of guinea-pigs, mice, and rats by the administration of lead, these structures gave a negative Feulgen reaction (Blackman, 1936)

5 Intranuclear inclusions developed in nerve cells after prolonged electrical stimulation of nerves in turtles, cats, and monkeys (Heinbecker and O'Leary, 1930)

6 Soaking nerve tissue in hypertonic saline produced nuclear inclusions (Davenport *et al.*, 1931).

7. Administration of irradiated ergosterol produced intranuclear inclusions in the kidneys of rhesus monkeys, and in the parotid and submaxillary glands of cebus monkeys (Cowdry and Scott, 1935 *a*)

A number of authors has described intranuclear inclusions in healthy young children (e g., Goodpasture and Talbot, 1921, Farber and Wolbach, 1932, Kuttner and Wang, 1934, Pappenheimer and Hawthorne, 1936; for other references see VonGlahn and Pappenheimer, 1925). The patients were usually infants, sometimes still-born fetuses were examined. The inclusions occurred in the bile ducts, intestine, kidneys, liver, lungs, pancreas, parotids, thyroid, and respiratory tract. Farber and Wolbach found both nuclear and cytoplasmic inclusions in 12 per cent. of submaxillary glands examined in infancy. Similar intranuclear bodies were found by Kuttner and Wang (1934). Recent authors describing inclusions in the organs of infants are Cappell and McFarlane (1947) and Kalfayan (1947).

Acidophilic intranuclear inclusions appeared spontaneously in cultures of human fetal leptomeninges, and in fibroblast cultures from human fetal lung (Fischmann and Russell, 1940).

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[1] Many of these inclusions have been personally studied by Cowdry (1934)

It may be said that virus inclusions usually give negative Feulgen and MacCallum tests, resist solution in hydrochloric acid, do not react to tests for mitochondria, the Golgi apparatus, and the centrosome, do not give a positive oxidase test, and frequently stain vitally with cresyl blue.

2. *Electron microscopy* has been used by Boswell in conjunction with us, and it appears that excellent detail can be made out, the granular nature of the inclusions of ectromelia, for example, being shown to be due to the presence of elementary bodies (Boswell, 1947).

3 *Tissue culture and egg culture.* Viruses may be added to the cells of a tissue culture, and the development of characteristic inclusions studied in wet, or preferably stained, preparations; the slide technique of growing cells is probably the best suited to this purpose (see also Ch XII). By such means the inclusions of herpes febrilis, lymphogranuloma inguinale, psittacosis, and vaccinia have been studied.

Viruses may be inoculated in fertile eggs (see also Ch. XIII); after an appropriate period of growth, the chorio-allantois is removed, and histological preparations made By this technique, the inclusions of herpes, lymphogranuloma, psittacosis, and vaccinia have been studied Agents of the psittacosis group produce inclusions in the cells of the yolk sac after local injection Andrewes (1930) has shown that the addition of immune serum prevents the development of herpetic inclusions (Lipschütz bodies). Grafts of human skin can be placed on the chorio-allantois and infected with virus, by this means the intranuclear inclusions of zoster have been studied (Goodpasture and Anderson, 1944). Human chorion and amnion have also been grafted, and the inclusions of herpes, variola, and vaccinia studied (Goodpasture and Anderson, 1942) Fowl skin has also been grafted and the inclusions of fowl-pox studied (Goodpasture and Anderson, 1940)

4. *Animal inoculation* Inclusion bodies are, of course, frequently studied experimentally in the tissues of infected animals, a particular application of this method has been used in the study of life cycles For example, in the study of psittacosis, mice were injected with virus in the peritoneal cavity, animals were killed at short intervals thereafter, and preparations made from the spleen (Bedson and Bland, 1932, see Ch LXXI) Working with lymphogranuloma inguinale, Findlay *et al*, (1938) injected mice intracerebrally, in this case also the animals were killed at short intervals thereafter In both these studies it was possible to identify a life cycle by examination of successive specimens

5. *Micro-incineration* Of recent years, it has been found that important information can be yielded by subjecting tissue containing virus inclusions to a very high temperature, after incineration, slides are examined microscopically for the nature of the residual ash Using this method, Scott (1930) found that the intranuclear inclusions of salivary gland disease of guinea-pigs left little or no ash, whereas nucleoli were found to be rich in ash A method of distinguishing between intranuclear inclusions and nucleoli is thus available Similar appearances are presented, after incineration, by the intranuclear inclusions of yellow fever (Cowdry, 1933), herpes (Rector and Rector, 1933), and Rift Valley fever (Horning and Findlay, 1934)

Danks (1932), studying intracytoplasmic bodies by micro-incineration, found that the Bollinger body of fowl-pox contained calcium salts, and was composed of numerous smaller granules, or Borrel bodies. Covell and Danks (1932) studied the Negri body, which yielded a compact white ash composed mainly of calcium

6 *Ultracentrifugation* This method was used by Lucas and Herrmann (1935) in their study of the Lipschutz body of herpes febrilis, they used the ultracentrifuge of Beams, Weed, and Pickels (Ch VI) A rabbit's cornea was excised, and cut into small pieces, 30 to 48 hours after inoculation with virus. The tissue was centrifuged for 45 to 60 minutes at 65 to 70 lbs air-pressure, fixed, and histological preparations were made The herpetic inclusions were heavier than chromatin, and passed to the pole opposite to that where the chromatin was collected

METHODS USED IN THE STUDY OF INCLUSIONS

1. *Microscopic examination.* Inclusion bodies may be examined in wet preparations by the darkground microscope, with a view to detecting granularity, infrared microscopy has also been employed. Both these methods have shown, for example, that the Negri body of rabies is a granular structure, and not homogeneous, as it appears in stained films Himmelweit (1938) has employed a very delicate microscopical method (see p. 343); he studied *in situ* by the Heine Ultropak (Leitz) microscope the chorio-allantois of ducks' eggs infected with vaccinia (and ectromelia). He found that in infected epithelial cells there were large numbers of granules, which increased in number, and were always contained in a matrix. There is little doubt that the granules were virus elementary bodies, and that the whole accumulation represented a Guarnieri body. Another method of studying vaccinia has been adopted by Merling (1940 *a*, *b*, 1943, 1945). The rabbit cornea is scarified and inoculated with vaccinia Later, cells are detached from the cornea by shaving or scraping with a knife. Cells are then placed in physiological fluid on a slide and sealed. Observation is then carried out by darkground for prolonged periods. EB's appear to enter the cell, coalesce to form Guarnieri bodies, and these rupture with the escape of EB's. Extracellular cysts, undergoing a life cycle are also seen. The observations of Himmelweit and Merling are strongly in favor of the vaccinial inclusion being a virus "colony."

As a general rule, however, inclusions are examined microscopically only after staining. Staining methods are applied for two main reasons.[1] First, as a means of demonstrating accurately the morphology of the body in question Secondly, stains and other reagents are applied to test, as far as possible, the composition of the inclusion, these tests are as follows·

(*a*) The Feulgen technique is performed as a test for thymonucleic acid. Virus inclusions usually give a negative Feulgen reaction, whether they occur in the cytoplasm or the nucleus. Lépine and Sautter (1946) have investigated a number of nuclear and cytoplasmic inclusions Thymonucleic acid was demonstrated by a positive Feulgen reaction, in rickettsiae and the inclusions of LGI and psittacosis, as well as in Guarnieri bodies (see also p 341). No thymonucleic acid was detected in the inclusions of rabies, Borna disease, yellow fever, herpes, pseudorabies, guinea-pig pneumonia, or ectromelia The bulk of inclusions probably contained no ribonucleic acid

Ingested nuclei may sometimes resemble virus inclusions, but their true nature can be detected by this reaction, for example, the so-called Gamna-Favre bodies of lymphogranuloma inguinale give a positive reaction, showing that they are of nuclear origin

(*b*) MacCallum's test detects the presence of masked iron, a positive reaction signifying the presence of nuclear chromatin

(*c*) The application of hydrochloric acid usually dissolves nuclear chromatin, but leaves inclusions intact

(*d*) Stains for mitochondria, the Golgi apparatus, and the centrosome prevent smaller forms of inclusion body from being confused with these normal intracellular structures.

(*e*) The oxidase reaction detects the presence of granules of leukocytes, which give a blue color Sometimes a flattened leukocyte may simulate a fibroblast with eosinophilic inclusions, sometimes also histiocytic cells may ingest the granules of degenerated leukocytes In both these cases the oxidase reaction shows that the intracellular structures are not true virus inclusions, but only leukocytic granules

(*f*) Intravital staining is sometimes carried out Cresyl blue solution is applied to wet preparations of tissue containing inclusions, which take up the stain selectively, to appear distinctly blue.

[1] For the technique of staining, a textbook of histology should be consulted

myelitis and zoster, we would reserve our opinion until further and more definite reports are available.

To conclude, we would summarize our views on the specificity of inclusion bodies in the following terms Certain inclusions, both cytoplasmic and nuclear, are so constantly found in virus infections that they must be regarded as specific structures produced by the respective virus agents There are, however, certain less definite inclusions, which have not been so thoroughly studied. We would not place these in the same category as those just mentioned, and we consider that their presence may be merely fortuitous, more particularly as it is evident that bodies morphologically resembling those of virus diseases can occur in conditions where the presence of any virus is improbable. The mere finding of an inclusion in a cell does not indicate a virus infection, the inclusions must occur constantly in cases of the disease, and be associated with the infectivity of the tissue in which they occur, before the possibility of a virus infection can even be considered

THE STRUCTURE OF INCLUSIONS

Any discussion on the structure of inclusions at the present day must take note of one outstanding fact. That is, that two typical intracytoplasmic inclusions—those of ectromelia and fowl-pox—have been isolated by microdissection, and found to be infective, further, one typical intranuclear inclusion—the Lipschutz body of herpes—has also been found to be infective.

There are two main theories in regard to the composition of inclusions In the first place, it is suggested that inclusions are formed from the cells of the host; that is, the inclusions are reactionary products For example, it has been variously suggested that certain inclusions may be formed by a process of degeneration of the cytoplasm or of the nucleus, that they are formed from extruded chromatin or nucleoli, that they are formed from the Nissl substance or mitochondria, or that they are formed from hypertrophied neurofibrils It is possible that the well-known Negri body is formed from nuclear material, or else from neurofibrils, but we do not believe that many other common types of inclusion can be satisfactorily explained by this theory. In the second place, it has been suggested that inclusions are intracellular colonies of virus elementary bodies We believe that this theory explains the inclusions of ectromelia, fowl-pox, molluscum contagiosum, variola-vaccinia, psittacosis, trachoma, inclusion conjunctivitis, pneumonitis, lymphogranuloma inguinale, and herpes febrilis. It is probable that the most feasible explanation of the composition of the majority of inclusions is that elementary bodies first enter the cell, sometimes reaching the nucleus, and then proliferate to form a colony. The cell itself probably reacts and produces a covering which gives the inclusion a more or less homogeneous appearance, especially is this so in the trachoma group of inclusions This theory was really advanced many years ago by von Prowazek (1907), who held that inclusions were formed from living organisms embedded in a matrix produced by the cell He coined the term *Chlamydozoa* to express the idea of organisms being clothed by a mantle of cellular protoplasmic material Later, Lipschutz (1921) regarded nuclear inclusions as reactionary products with which were associated virus particles

The problem of the biological nature of inclusions calls for much further study, at the present, too much use has to be made of analogy More experimental data, especially such as can be yielded by micromanipulation and electron microscopy, are much required

SOME STAINS FOR INCLUSION BODIES

We do not propose to discuss this question fully, as the methods are readily available in any manual on histology, but we give details of certain methods that we have found satisfactory Some methods of staining Negri bodies are described

7. *Micromanipulation* There is one method, beyond all others, of deciding whether an inclusion is a purely degenerative structure derived from the host, or whether it is infective, and derived from the parasite; that method is to isolate an inclusion by micromanipulation and then, *if possible, inject it into a susceptible* animal, eventually reproducing the infection.

The standard work on these lines was carried out by Woodruff and Goodpasture (1929), who isolated a fowl-pox inclusion by micromanipulation; it was then washed in saline, and finally injected into an animal. The washed inclusion reproduced the disease, while the saline used to wash the body *proved noninfective.* They further showed that an inclusion contained numerous elementary bodies. Baumgartner (1935 *a*) confirmed these results, and also worked with the inclusions of ectromelia of mice. She was able to reproduce the infection with a single washed ectromelia inclusion Baumgartner (1935 *b*) has also carried out important work on the intranuclear Lipschutz body of herpes The isolated body *proved infective* for the cornea, whereas the saline in which it was washed proved negative.

van Rooyen (1938) has applied this technique in another direction, he has carried out microdissection of the inclusion of molluscum contagiosum, and has shown that the body can be removed from the epithelial cell, and that it contains numerous elementary bodies embedded in a sticky matrix

The significance of the above results is undoubted, it has been shown that two very typical intracytoplasmic inclusions, and one typical intranuclear inclusion, are infective, and must, therefore, contain the viable virus particles These results have greatly influenced modern views on the nature of inclusions, and many feel that it is only a matter of time before other well-known inclusions, e.g., those of vaccinia, rabies, and yellow fever, will be treated in the same way and found to be infective

THE SPECIFICITY OF VIRUS INCLUSIONS

With regard to intracytoplasmic inclusions, we take the position that a number are absolutely specific structures that do indicate the presence of a virus. They are so characteristic in their morphology that they cannot be confused with other structures Further, they are associated with the infectivity of the tissue in which they occur, and many develop in infected tissue cultures and eggs. Such inclusions are those of rabies, variola-vaccinia, molluscum contagiosum, lymphogranuloma inguinale (Miyagawa's bodies), psittacosis, pneumonitis, ectromelia of mice, fowl-pox, trachoma, and inclusion conjunctivitis.

There are, however, certain other types of intracytoplasmic inclusion which have not been so fully studied and may be nonspecific, being unconnected with *the causal agent It is possible, however, that further study may transfer some of* these structures into the group just mentioned above *Bodies, the specificity of* which we do not yet regard as established, are Da Fano's or Nicolau's bodies of herpes febrilis, Da Fano's or Dawson's bodies of encephalitis lethargica; Taniguchi's bodies of Japanese encephalitis, eosinophilic bodies in warts, basophilic bodies in measles

With regard to *intranuclear inclusions the position is rather different For, first,* intranuclear inclusions occur in a variety of nonvirus infections, in healthy animals and human beings, and can be produced experimentally Second, there are only two common appearances presented by intranuclear inclusions—Types A and B Many nuclear inclusions, however, are found so uniformly in the particular disease, and are so *constantly associated* with the *infectivity* of the tissue in which they occur, that they may be regarded as specific structures due to the virus in question Inclusions which we so regard are those associated with yellow fever, herpes (Lipschutz bodies), Rift Valley fever, varicella, "B" virus, and virus III With regard to the intranuclear inclusions of Nicolau in herpes, and the inclusions of polio-

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in Ch. LXXVI and trachoma bodies in Ch. LXV. It should also be remembered that Giemsa's method can be used to demonstrate inclusions.

Mann's Stain

We have used this particular modification with success:

Stain. 1 per cent. methyl blue, *35 c.c.; 1 per cent. aqueous soluble eosin, 15 c.c.;* water, 100 c.c.

Method. Take the section to distilled water, and treat with iodine and thiosulphate if necessary. Apply Mann's stain, and leave for 10 minutes (when possible, leave for several hours). Wash and differentiate in tap water. Wash in alcohol, treat with xylol, and mount.

H. E. Hutchison (Glasgow) advises the substitution of methyl blue (CI 706) by soluble blue (CI 707) especially for rabies material received in formol, spirit is a preferable fixative for such material.

Laidlaw's Stain

Tissues should be fixed in acetic-corrosive sublimate fixative for the best results.

1. Take section to distilled water, and treat with iodine and thiosulphate. Stain with Weigert's iron hematoxylin for 5–10 minutes. Wash well with tap water to "blue," 3–5 minutes.
2. Treat with 0.25 per cent. acid-alcohol Blue with tap water. Wash with distilled water.
3. Stain with 1 per cent. acid fuchsin for 5 minutes. Rinse with distilled water.
4. Mordant with 1 per cent. phosphomolybdic acid, 2–5 minutes Wash with distilled water.
5. Differentiate in 1 per cent. orange G in 60–70 per cent. alcohol, alternating with water to watch progress.
6. Take to alcohol, treat with xylol, and mount in dammar.

Phloxin-tartrazine (Lendrum, 1939, 1947)

This method promises to prove very suitable for phloxinophilic cytoplasmic inclusions.

Stain by hemalum (Mayer's 1903–4 formula, see Lendrum and McFarlane, 1940) for the usual time Blue and wash. Stain with 0.5 per cent. phloxin (CI 778) in water containing 0 5 per cent. calcium chloride, for 30 minutes in a jar Rinse quickly in water, drain off, and replace by a saturated solution of tartrazine NS in cellosolve (ethylene glycol monoethyl ether) from a drop bottle, and control microscopically, this acts as a differentiator. The tartrazine is very soluble in water and is easily removed by washing When the desired degree of removal of red (phloxin) has been reached, a further brief treatment with tartrazine is followed by a rinse with 60 per cent spirit, 95 per cent. and absolute alcohols, and xylol

Lendrum found structures to vary considerably in their phloxinophilia, and those found to be strongly phloxinophilic included the polymorphous cytoplasmic inclusions of warts, Kurloff bodies of the guinea-pig, and cowpox inclusions in egg membrane. The method well merits further trial

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CHAPTER XI

SEROLOGICAL TECHNIQUE IN THE STUDY OF HUMAN VIRUS DISEASES

Recent studies have shown that the immunological characteristics of the filtrable viruses are analogous to those of the visible bacteria, and many serological phe- [illegible] neutralization tests— [illegible] minor modifications of [illegible] to render such tests [illegible] lty, in fact, involves [illegible] t, while bacteria are [illegible] in presence of living cells. Therefore, for certain serological tests it may be necessary to separate the virus agents from the tissues containing them This may be done by differential centrifugalization and repeated washing of tissue extracts, so that suspensions of elementary bodies are prepared. Although relatively pure suspensions of elementary bodies are thus obtainable it is impossible to eliminate all traces of antigen derived from the parasitized cells. It is therefore necessary to include a special control in the reactions, this consists of an extract of normal tissue which has been prepared from the corresponding organ of the same species as that of the animal infected with the virus under investigation This extract is prepared exactly as is that of the infected material.

For example, if an elementary body suspension obtained from rabbit's testicle is to be agglutinated with antivaccinial serum, then the antigen control should consist of a saline extract of normal rabbit's testis, which has been subjected to the same treatment as the infected tissue.

The serum controls demand special attention, and sometimes two are required for each serum tested. For example, if an antivaccinial serum has been prepared by immunizing a rabbit with vaccinia virus cultivated on the chorio-allantoic membrane of the chick embryo, then there should be a control with one sample of serum derived from another rabbit after immunization with normal chick embryo and a second from a normal rabbit. In dealing with homologous tissue, e g., when a rabbit is actively immunized with vaccinia virus cultivated in rabbit testis, a single control consisting of normal rabbit serum suffices

Innumerable modifications of the various common serological techniques have been used by virus workers It is quite impossible for us to describe the technical details of more than a few of the more representative and orthodox methods. References to other techniques will be found in the appropriate chapters, where also *the phenomenon of hemagglutination-inhibition by immune serum will* be discussed

THE PREPARATION OF SPECIFIC IMMUNE SERA

Vaccinia

Rabbits can be immunized with comparative ease against vaccinia virus. A normal animal is inoculated subcutaneously with 0 25 c c. of a 1 1,000 dilution of vaccinia virus contained in desiccated testis. After 3 to 5 days a cutaneous reaction develops at the site of injection, and thereafter the condition may either generalize (see Douglas, Smith, and Price, 1929), or else the lesion may remain localized and subsequently retrogress, depending on the virulence of the strain of virus employed Recovered rabbits are resistant to reinfection, and their immunity may be exalted by repeated doses of virus, which may be administered either intravenously

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A third horse was injected with 6 subcutaneous doses of ferret lung suspension containing Shope's strain of hog influenza virus, the dosage varying from 30 to 50 c c. over a month. After one month's rest, hyperimmunization was continued and the animal received, without signs of ill effects, the remaining injections consisting of a mixture of human and porcine strains of virus cultivated in ferrets.

Antisera are readily produced in rabbits by the inoculation of two doses at weekly intervals of, for example, 5 c c. of virus-infected allantoic fluid. Antibodies also develop quickly in ferrets or mice on recovery from infection (see Chapter LXII).

VIRUS NEUTRALIZATION TESTS

Tests for virus neutralizing antibodies have been employed for three different purposes, namely (1) to assess the potency of therapeutic antisera with a view to their standardization, (2) for detecting the presence of virus neutralizing antibodies in human or animal sera for diagnostic or epidemiological purposes, and lastly, (3) to identify viruses or to investigate their antigenic structure. The general principle on which these tests are based is the same, for in the majority of instances the serum to be tested is mixed with the virus in suitable proportions, incubated for a short time, and inoculated into a susceptible animal or an egg In some tests the amount of virus used is kept constant and different dilutions of serum are added, in others the amount of serum is kept constant and the virus diluted instead.

The criteria by which individual neutralization tests are interpreted vary greatly. In general they depend on the susceptibility of the animal used, e g, in the case of neutralization tests with Rift Valley virus, as mice are employed for neutralization tests and as this virus is highly pathogenic to them, the results are interpreted according to the number of mice which survive. Should, however, a virus which is less infective to mice be employed, then after a definite interval of time all animals which do not die must be killed and examined for evidence of lesions For example, in such tests with psittacosis, the spleen of each mouse must be stained and examined for the presence of elementary or inclusion bodies before it is possible to tell whether the serum has, or has not, neutralized the virus, and in the case of tests with influenza virus the lungs of the animals are searched for evidence of consolidation.

The mouse protection test for yellow fever immune body is conducted on a different principle, thus before the serum-virus mixture is introduced intraperitoneally, the animal is given an intracerebral dose of sterile starch solution to induce the spread of virus from the brain capillaries to the brain itself, and the presence or absence of immune body in the serum tested is indicated according to the number of mice which develop fatal encephalitis (see Ch XLIII for the technique of this and other neutralization tests in yellow fever).

With a certain number of viruses it has been possible to utilize cutaneous skin reactions for titrating the antibody content of immune serum, so that several experiments can be performed simultaneously on one animal Gordon (1925) used this method for his studies on vaccinia virus and we have used it for our experiments on rabbit myxoma and Shope's fibroma viruses (van Rooyen, 1938, van Rooyen and Rhodes, 1938) The chorio-allantoic membrane of the developing chicken embryo can also be used for neutralization tests, and Burnet (1937, *et al*, 1937) showed that after inoculating the serum-virus mixture on the membrane and counting the number of pocks produced, the neutralizing powers of the serum could be estimated Certain antibodies can be titrated by inoculation of serum-virus mixtures in the allantoic cavity (influenza, see Ch. LIX), or yolk sac (equine encephalomyelitis, see Ch XCVI)

It is now customary in these tests to take the titer of the serum as the 50 per cent end-point, the point where equal numbers of animals inoculated with serum-virus mixtures show, or do not show, the lesions characteristic of the particular

or intraperitoneally (see Gordon, 1925; Craigie and Tulloch, 1931, Craigie and Wishart, 1934 *a*; Smith, 1930).

Hyperimmune sera contain agglutinins, precipitins, complement fixing and virus neutralizing antibodies. There is no standard method for estimating accurately the potency of such sera, but about 1 c.c of an active antivaccinial serum should protect against 1,000 minimal infecting skin doses, according to Andrewes (1928).

Horses. Ledingham, Morgan, and Petrie (1931) actively immunized a horse against vaccinia virus, with the aim of studying the properties of such serum as well as providing themselves with antivaccinial serum for use in cases of post-vaccinal encephalitis or smallpox.

The animal was first immunized with formolized diphtheria toxin and tetanus toxin of which 5 doses were given. Subsequently it was injected repeatedly with glycerolated calf lymph (Government strain), and during this period it was given a routine dose of tetanus toxoid every 2 or 3 months. The dosage of virus consisted of an initial intradermal inoculation of a 1·10 dilution of lymph, which was scarified into an area of shaved skin about a foot square, situated on one side of the animal's neck. Fifty hours later, a crop of about 200 vesicles developed, then gradually subsided and healed, so that after 35 days the skin had returned to its normal condition The animal was now hyperimmunized by a further inoculation of 10 c.c. of lymph given subcutaneously. This was followed 8 weeks later by 71 c.c. intravenously, a month afterward by 40 c c intravenously, and finally with 130 c.c. given intramuscularly The total bulk of glycerolated calf lymph introduced was 282.5 c.c. administered over a period of 9 months

Sample bleedings, varying in amount from 0.75 to 2.2 litres, were made at different periods during the course of immunization, and the last quantity of blood was withdrawn about 3½ months after the final intramuscular dose.

Herpes

Guinea-pigs. Antivaccinial or antiherpetic immune serum may be prepared by injecting these animals repeatedly with large doses of live virus Bedson and Bland (1929) recommended that they be given 6 to 8 doses of 1.0 to 2.0 c.c. of a 10 per cent. suspension of virus material, administered intraperitoneally, and sera prepared thus were shown to contain complement fixing antibodies.

Psittacosis

Guinea-pigs are comparatively resistant to psittacosis virus, and Bedson (1933) has successfully immunized them against this agent. A mouse was first inoculated with virus, its spleen removed and extracted with saline to make a 10 per cent. suspension of tissue. Guinea-pigs received 2 to 3 c.c. of the 10 per cent extract administered intraperitoneally at weekly intervals, with periodic rests of 1 to 2 months, each of these animals received from 10 to 15 injections, and their sera contained both virus neutralizing and complement-fixing antibodies

Influenza

The horse has been shown to be resistant to this agent, influenza virus administered intratracheally in 4 animals yielded negative results (Laidlaw, Smith, Andrewes, and Dunkin, 1935) The animals were hyperimmunized by giving each horse 12 subcutaneous injections of about 15 c c in bulk, prepared from infected ferrets' nasal mucosae, over a period of 4 months This was followed by 6 further injections of virus contained in ferret lungs, over a space of 8 weeks, at the end of which the horse's serum was found to neutralize influenza virus.

A second horse was immunized with 50 c c doses of ferret lung emulsion for 2 months, the last 3 injections of virus consisting of a mixture of both the human and porcine strains. The animal appeared to exhibit considerable reactions to the latter inoculations

tions, one mouse cage should be used for each dilution, and sick mice promptly removed Mice are observed for 10 days with the equine viruses, unless recently isolated, when 14 days suffices, as it does for St. Louis virus. For Russian and Japanese encephalitis viruses 3 weeks are required In tests with equine viruses, deaths occurring within 24 hours are regarded as nonspecific. With the other encephalitis viruses, deaths up to 48 hours are regarded as nonspecific, deaths occurring from 3 days and later should be charted as being specific.

Where additional information is required, extraneural tests may be used, or serum may be used in varying dilutions, with 10 or 100 LD50 of virus or with varying amounts of virus

Advantage may be taken of the increased susceptibility of baby mice to the extraneural injection of virus to demonstrate antibody increases Thus Lennette and Koprowski (1946) have used the extraneural test in demonstrating the development of antibodies in man following vaccination with Western equine vaccine. Mouse brain suspension was used as antigen Undiluted sera were mixed with equal volumes of virus dilution and tubes were incubated at 37° C. for one hour. Mixtures were then inoculated in 0 03 c c. quantities subcutaneously in 3-day-old mice, and cerebrally in 28-day mice. The 50 per cent. mortality end point titer of virus in the presence of pre- and post-vaccination sera was calculated and expressed as "effective virus titer"; the difference between the logarithms of the two titers was expressed arithmetically and designated "neutralization index" representing the number of LD50 of virus neutralized. When the post-vaccination sera were tested by the cerebral route, only a slight response could be demonstrated By means of the extraneural test, however, it was possible to measure a definite response

Poliomyelitis

Kolmer and Rule (1935) recommended the following method for detecting the presence of virus neutralizing antibody in human serum The spinal cords from severely paralyzed monkeys were removed, stored in 50 per cent. glycerol in the refrigerator for a month, and a saline suspension containing about 10 m l d of virus per 0 5 c c. was prepared as follows 1 gm. of the spinal cord was washed and ground up in 20 c c of saline to make a 5 per cent suspension, centrifuged at 1,500 r p m for 3 minutes, and the supernatant fluid used for the experiment Then 0 5 c c of this material was mixed with 0 5 c c of patient's serum, placed in the water bath at 37° C for 2 hours, and 0 5 c c injected intracerebrally in a *M. rhesus* monkey. The control test was carried out by inoculating another monkey with a similar suspension of virus, to which saline had been added instead of serum

Schaeffer and Muckenfuss (1940) investigated the virus neutralization test very thoroughly and made a number of suggestions for improving the technique, using monkeys More recently, virus neutralization tests have been carried out in mice, using the Lansing strain (Armstrong, 1941), by this method enough animals can be used to give a reliable result, but it is not clear to what extent antibodies to this strain develop in man (see Ch LXXXVIII) Olitsky and Findlay (1946), using the MEF strain, mixed equal parts of neat serum and serial dilutions of virus, after holding at 37° C for 2 hours, the mixtures were inoculated cerebrally in mice A serum was regarded as positive if it neutralized 100 lethal doses

Rift Valley Fever

Neutralizing antibodies to this agent have been demonstrated in the sera of human beings recovered from an attack of the disease (Findlay, 1936)

A 10^{-1} dilution of infected mouse liver was diluted with saline to form dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, to each of these was added an equal volume of immune serum, and to a similar series of dilutions normal serum was added to serve as a control The two sets of mixtures were incubated at 37° C. for 4 hours, and

virus. For the calculation, a sufficient number of animals or eggs, usually 6 per dilution must be used, and the end-point worked out by a method such as that of *Reed and Muench (see Ch. VIII).*

Tests with Neurotropic Viruses

Olitsky and Casals (1947), in an authoritative paper, discuss the general principles involved in the testing of antisera for virus neutralizing antibodies against the encephalitis-producing group of
of high and uniform susceptibil
sera may be expressed by means
by calculating the LD 50 of control and test serum-virus mixtures separately, subtracting, and expressing as the antilogarithm of the difference. Thus if the LD 50 of the control serum-virus mixture is 6 5 and of the test serum mixture 4, the difference is 2 5, and the neutralization index is 320 (the antilog of 2.5). The number obtained by this calculation can, however, show only the order of magnitude of, and not the precise amount of antibody. It is recommended that for indices of 1–50 the result is expressed to the nearest whole number, 50–100 to the nearest 10, 101–1000 to the nearest hundred, 1001–10,000 to the nearest thousand, and so forth. They allude to the observations of Morgan and of Whitman that samples of serum stored at 4° C. may partly lose their neutralizing capacity, whereas sera immediately frozen retain neutralizing potency. It appears as if a complement-like heat-labile substance is responsible for at least a proportion of the virus-neutralizing effect. In all tests where acute and convalescent phase sera are compared, it is essential that both samples are stored in the same way, preferably frozen, otherwise false results will be obtained Or, as recommended by Whitman, sera heated to 56° C. for half an hour may be used, thus testing only the heat-stable neutralizing antibody.

As regards the technique of neutralization tests, several other precautions are recommended (1) Serum should be obtained with sterile precautions, several hours after a meal. After clotting for half an hour at room temperature, the serum is separated by centrifugation. Phenyl mercuric borate, 1 50,000, can be used as preservative. Hard glass ampules should be filled one-third full, and frozen on CO_2 snow

(2) The virus antigen is obtained by pooling the brains of 10 mice ill, but not dead, from infection, the brains are ground in a blender to a 20 per cent suspension in 50 per cent. or neat normal rabbit serum heated to 56° C for half an hour The suspension is distributed in glass ampules in 1 or 2 c c amounts After sealing, the ampules are quickly frozen in CO_2 and 95 per cent alcohol, and are kept frozen with CO_2 snow From time to time, the strain of virus should be checked and compared for activity, maintenance of titer, and general reliability

(3) The test recommended by Olitsky and Casals has been employed for over 20 years, and includes various modifications When thawed, the frozen virus antigen represents a 1 in 5 dilution Dilutions of 1 50, 1 500 and so forth are prepared, using as diluent 10 per cent rabbit serum in isotonic sodium chloride. The tubes receiving virus are kept in ice From each of the dilutions that are to be tested, 0.2 c c. is to be placed in each of two tubes. To one series is added 0 2 c c of neat negative human serum (or 10 per cent normal rabbit serum), this serves as the control series In the test series, 0 2 c c of unknown serum is added instead The tubes are incubated in the 37° C water bath for 2 hours, and placed in ice water prior to cerebral injection, at least 5 mice are used for each dilution For the test serum, dilutions of virus should be employed which indicate no neutralization or neutralization of at least 1,000 LD50 Thus if the LD50 of virus is usually at 10^{-8}, the control series should be made up with virus dilutions of 10^{-5} to 10^{-9}, and test sera with 10^{-4} to 10^{-7} The highest dilutions of the test series should be injected first, and the controls last To obviate the risk of secondary contact infec-

incubating the mixture at 3 ... tor overnight. The excess ... the suspension through a c... retained the virus, or else ... absorbed sera were now tested for the presence of virus neutralizing antibodies by admixture with dilute virus filtrates of about 100 m.i.d. in potency, and the result was compared with serum which was unabsorbed, but had been subjected to similar treatment. Each serum-virus mixture was incubated at 37° C. for 2 hours, and thereafter tested for infectivity by intranasal inoculation of mice. Control tests were also incorporated in the series, and it was shown that the virus neutralizing antibodies could not be absorbed by normal lung tissue From these experiments Smith and Andrewes were able to show that absorption of a serum with a homologous strain of virus removed only its appropriate antibodies, leaving the others intact.

AGGLUTINATION OF ELEMENTARY BODIES

Vaccinia

Craigie (1932) has employed the following method for obtaining a suspension of vaccinial elementary bodies·

A piece of bronze wire gauze (100 mesh) is used for scarifying the shaved back of a rabbit, a strip of the gauze (0 7 cm. by 10 cm) is held with a pair of Kocher forceps, and the virus suspension is placed on the skin of the rabbit which is then inoculated by drawing the rough edge of the gauze over the animal's back. Three days later the rabbit is exsanguinated, killed, the skin removed, cleaned with ether, pinned out on a board, and the pulp scraped off with a scalpel into two successive 10 c.c. volumes of chilled citric acid (0 004 M) one part and phosphate buffer (of pH 7 0) one part diluted 1 50 in distilled water

The pulp suspension is not ground up but is thoroughly shaken, centrifuged at 3,500 r.p.m. for 10 minutes, and the supernatant fluid removed and set aside. The deposit is once again washed in dilute buffer solution, and the supernatant fluids obtained after first and second washings are pooled. The mixture is now placed in flat angle centrifuge test tubes of 4 mm. internal diameter, and centrifuged at 3,500 r p m for 60 minutes at an angle of 28° from the vertical The elementary bodies deposited at the end of this interval are resuspended in fresh buffer solution and subjected to two further washings. The final suspension is dispersed by thorough shaking and once more centrifuged at 3,500 r p.m for 10 minutes on the ordinary centrifuge, in order to remove coarse tissue particles The supernatant fluid containing the elementary bodies is now removed, placed in a glass-stoppered bottle, excess anesthetic ether added, and stored in the ice chest until required The ether can be removed before using by placing a small quantity of the suspension in a partial vacuum Craigie and Wishart (1934 *a*) state that the yield of Paschen bodies obtained from a rabbit in 15–20 c c of dilute buffer has, when fresh, an intradermal infective titer of 10^{-8}

The density of the elementary body suspension used as antigen is standardized to match the opacity of a kaolin suspension corresponding to tube No 2 of the McFarlane standard. The tubes are compared with each other by placing them against a dark ground illuminated from behind at an angle of 45°.

A series of serum dilutions 0 25 c c in bulk is made in 0.85 per cent sodium chloride containing 1 50 citric acid phosphate buffer of pH 7 0 The elementary body suspension (*vide supra*) is diluted to twice the standard opacity in distilled water containing 1 50 of this buffer, and 0 25 c c. added to each serum dilution The mixtures are incubated in a water-bath at 50° C. for 16 hours and the results read macroscopically By means of agglutinin absorption tests Craigie and Wishart (1935) have demonstrated th... agglutinins in antivaccinial

0.4 c.c. of each serum-virus mixture was inoculated intraperitoneally in mice. The results showed that an immune serum was able to neutralize a 1:10 or higher dilution of virus, whereas normal serum was incapable of doing so, and the animals accordingly succumbed to infection in 2 to 5 days' time.

Psittacosis-LGI-Pneumonitis

Differentiation between the closely related members of this group can be achieved by means of antisera prepared in chickens; serum-virus mixtures are inoculated in mice nasally or cerebrally, according to the agent (St John and Gordon, 1947).

THE ABSORPTION OF VIRUS-NEUTRALIZING ANTIBODIES

Vaccinia and Herpes

Smith (1930) demonstrated that rabbit's testis infected either with vaccinia or herpes virus was capable of absorbing the homologous antibody from an immune serum, whereas normal rabbit kidney failed to absorb immune body from an antivaccinial serum

The specificity of the phenomenon was demonstrated by cross absorption experiments in which vaccinial testis failed to absorb herpetic antibody, and herpetic testis failed to absorb vaccinial antibody. It was also shown that each virus selectively absorbed its homologous antibody when added to a mixture of antivaccinial and antiherpetic serum. Vaccinial testis suspension, in which the virus was destroyed by heating to 58° C or 60° C. for 1 hour, showed greatly reduced power of antibody absorption

Five to 7 gm of vaccinial testis were minced with scissors in a sterile mortar with 10 c.c. of a 1 10 dilution of immune serum, and the suspension transferred to test tubes, 5 to 7 gm of normal testis (or kidney) were likewise treated and utilized as the control, 0.5 c.c of pure anesthetic ether was added to each tube which was hermetically sealed and stored for 3 days at 37° C., in order to destroy any contaminating bacteria.

After 3 days, the tubes were opened to allow the ether to evaporate, and were centrifuged at 3,000 r p m for ½ to 1½ hours The supernatant fluids were clarified by preliminary filtration through filter paper, or paper pulp, and then passed through L_2 candles under a negative pressure of 40 cm of mercury The final filtrates thus represented *(a)* immune serum treated with testicular virus, and *(b) immune serum treated with normal control tissue*

The method of antibody titration was as follows in order to compare the antibody content of any two sera, various dilutions of virus (i e, *of a suspension of* herpes or vaccinia virus contained in testis) were added to equal quantities of each of the sera, 0.2 c c of the mixtures was then injected intradermally in the shaved skin of a rabbit, using one half of the animal's back for each set of dilutions

Reactions were read on the third, fourth, and fifth days after inoculation By measuring the size of the reaction produced by each dilution, and the titer to which this occurred, it was possible to compare the virus neutralizing properties

Influenza

Absorption tests *were utilized by Smith and Andrewes (1938) for their investigations* into the complex antigenic structure of influenza virus, and they showed that each strain of virus only absorbed from a homologous serum those antibodies which corresponded to the antigenic components of the absorbing strain. Technical details regarding the method of performing the test were as follows A dilution of serum capable of neutralizing 100 m i d of virus was added to a 5 per cent. suspension of fresh infected mouse lung, and absorption *allowed to take place by*

clean glass slide with a drop of serum. The slide was then slowly tilted to and fro over an electric light bulb. With positive sera, visible aggregates could be seen by the naked eye within 2–3 minutes, negative tests remained uniformly dense.

Nineteen sera, from pigeons suffering from latent ornithosis, positive by complement fixation, were also positive by the agglutination technique.

PRECIPITIN REACTIONS

Variola-Vaccinia [1]

The following products of vaccinia virus have been proved to be precipitable with antivaccinial serum· A precipitable substance in Seitz (EK) filtrates of vaccinal pulp divisible into two antigenic fractions consisting of a thermolabile (L) portion which is destroyed by heating at 56° C for 1 hour, and a thermostable (S) component which resists 95° C. for a similar period.

Both these antigens occur in the elementary bodies of vaccinia, and participate in the agglutination of these bodies with vaccinial antiserum (Craigie, 1932, Craigie and Wishart, 1936 *a*). The LS fraction is prepared as follows.

A Seitz (EK) filtrate is dialyzed in cellophane bags A 1/10 volume of Sorensen's hydrochloric acid-citrate buffer (Clark, 1928) of pH 4 45 is added to the dialyzed filtrate and the mixture centrifuged for 30 minutes. The deposit is drained and the fluid adhering to the walls of the tube dried with filter paper. The deposit is then suspended in Sorensen's citrate caustic soda buffer of pH 6 65, the volume used being 1/10 of the final volume desired. Distilled water is added to the amount of four times this volume, the tubes shaken, centrifuged, and the supernatant fluid set aside The deposit is extracted a second time with the same volume of buffer (pH 6 65) and distilled water as previously used. The two supernatants are pooled, extracted with ether, kept for 24 to 48 hours in the ice chest, recentrifuged to deposit large particles, the supernatant fluid removed and used as LS fraction.

The antigenic properties of the L and the S components have been demonstrated by immunizing rabbits with each of them. The sera thus obtained react specifically to produce a precipitate with the homologous fraction in each instance It has been pointed out that, whereas 56° C annuls the *in vitro* activity of the L componen [illegible] it must be heated [illegible] ent, on the other [illegible] 1936)

A third precipitable substance has been obtained by extraction of elementary bodies after they have been freed from other tissue products by repeated washing. This has been termed "dissociated antigen" since it is liberated from elementary bodies *in vitro* It also consists of thermolabile and thermostable soluble precipitable substances, both of which are similar to the soluble precipitable substances found in suspensions of fresh vaccine pulp (Craigie and Wishart, 1936 *b*)

Parker and Rivers (1935, 1936, 1937) have confirmed Craigie and Wishart's findings They succeeded in extracting a chemically stable, serologically active substance from dermal and testicular tissue infected with vaccinia by boiling it with 50 per cent ammonium sulphate, followed by dialysis against running water, precipitation with alcohol, and treatment with buffered solutions About 15 mg of a white substance were derived from about 200 c c of an extract of dermal vaccine virus, and this was susceptible to digestion with commercial trypsin or pepsin but resistant to crystalline trypsin and chymotrypsin, and possessed the characters of an alcohol-soluble protein which was not precipitated by boiling in a neutral aqueous solution. A dilution of 1 640,000 gave a precipitate with an antivaccinial S antigen serum (Craigie and Wishart, 1934 *a*), but produced no reaction in a serum from which the S agglutinin had previously been removed by absorption

[1] See also p 370

sera. The two corresponding agglutinogens differ in their relative stability to heat and other agents (see Ch. XXXIII).

Zoster and Varicella

Amies (1934) has demonstrated the relationship of these two viruses to one another by means of cross agglutination reactions

Vesicle fluid was collected from patients, not more than 60 hours after the beginning of vesiculation, with a sterile capillary pipette. It was diluted in physiological saline containing 2 per cent. sodium citrate, centrifuged at low speed to deposit tissue cells, then the supernatant fluid was removed and recentrifuged at 12,000 r.p.m. for 30 minutes

The deposit (which was frequently invisible to the naked eye) was resuspended in saline of pH 7.0 containing 0.25 per cent. formalin. After a final fractional centrifugation at low speed to throw down gross particles, the supernatant fluid was removed and the suspension standardized to approximate in density that of an arbitrarily chosen elementary body suspension.

The hanging drop method was used. Dilutions of convalescent serum derived from cases of zoster and varicella were prepared from 1.4 to 1 128 in saline, mixed with an equal volume of antigen, and placed on a coverslip which was inverted over a glass slide fitted with a metal ring. The mixtures were left at room temperature (average 65° F.) for 24 to 48 hours and then examined under the microscope, using a 2/3 in. objective and × 10 ocular for evidence of agglutination A positive test was signified by the presence of highly refractile, irregularly shaped clumps

Psittacosis

Bedson (1932) has demonstrated that the elementary bodies found in this disease can be agglutinated l infected mouse spleen by fractional deposited after the final washing by ɔo r.p m

The antiserum employed consisted of the sera of guinea-pigs which had been immunized with psittacosis virus obtained from infected guinea-pig tissues Two control sera were also used, the one consisting of the serum of a guinea-pig which had been inoculated with mouse spleen tissue, and the other antiherpetic serum also of guinea-pig origin. The hanging drop technique was used for the tests and the results were read after incubating the mixtures at 37° C. for 24 hours From these experiments, Bedson proved that a suspension of psittacosis elementary bodies could only be agglutinated by homologous antiserum, and not by herpetic antiserum nor by antiserum to normal mouse spleen.

Labzoffsky (1946) has introduced a test for the laboratory diagnosis of ornithosis, using EB's grown in the egg To obtain allantoic fluid rich in elementary bodies, it was necessary to use eggs in one of the first five serial passages after transfer from the mouse Allantoic fluid was harvested from embryos dying on the fourth day or later. Fluids containing numerous elementary bodies might titer 10^8 or 10^9 on intracerebral inoculation of mice

For the preparation of the antigen for rapid agglutination tests, strongly positive allantoic fluids were pooled and inactivated with 0.4 per cent formalin After 4 days at 4° C., the material was centrifuged to throw down the elementary bodies The sediment was resuspended to 1/100 of its original volume with 0.1M phosphate buffer pH 7.2, further diluted 1/5 with normal saline, and thoroughly redispersed After "settling" overnight at 4° C., the dense supernatant constituted the antigen for the rapid agglutination test

Complement fixing antigen was prepared by inactivation of strongly positive allantoic fluid for 20 minutes at 70° C., and was used undiluted and without further treatment, material so treated contained no living virus

The rapid agglutination test was performed by mixing a drop of antigen on a

increasing quantities of complement, ranging from 1 to 10 m h d Others have diluted the antigen and kept the remaining reagents constant in volume (Craigie and Wishart, 1936).

The sequence in which the reagents are added conforms to standard serological practice, antigen, serum, and complement being added in that order. These are incubated together for either 1 hour at 37° C, as advised by Ciuca (1929) and Laidlaw and Dunkin (1931), or else for 18 to 20 hours at 8° to 10° C., as advocated by Bedson (1928), Bedson and Bland (1929), and Craigie and Wishart (1936). Others, such as Hoyle and Fairbrother (1937), in their tests with influenza virus, incubate the mixtures for 4 hours at 37° C, followed by a further period of 24 hours at 0° C, as these workers have pointed out that since the fixation of complement by mixtures of antigen and antibody may be slow, prolonged interaction is necessary. Owing to the fact that complement deteriorates rapidly if maintained at 37° C. for longer than 2 to 3 hours, when fixation is performed over a period of 16 to 18 hours' duration, low temperature is obligatory (see also Thomson, Hazen, and Buchbinder, 1932). Thus, in a review of the literature dealing with this subject, Parker and Muckenfuss (1933) have suggested that the failure of the earlier investigators to demonstrate specific complement fixation with variola-vaccinia virus, can be attributed to the fact that the virus and serum were incubated for too short a time

Antigens Used in Complement Fixation Tests

1. Tissues and exudates can be employed as a source of complement fixing antigen. Thus, the vesicle fluid from cases of zoster fixes complement with antisera (Netter, Urbain, and Weissmann-Netter, 1924, Bedson and Bland, 1929) The test is extensively used in variola, with vesicle fluid or crusts the source of antigen (see below).

Vaccine calf lymph can be used as antigen with antivaccinial or variolous sera (Jobling, 1906, Dahm, 1909, Kolmer, 1916) The antigen is resistant to boiling (Glaser and Koref, 1928, Lurje and Wolkowitsch, 1928).

Simple tissue suspensions can be used Thus Bedson and Bland (1929) used a 5 per cent suspension of tissue infected with vaccinia or herpes in M/50 phosphate buffer, clarified by light centrifugation or prolonged standing A 5 per cent. suspension of mouse spleen infected with psittacosis was used by Bedson (1933). A suspension of monkey parotid has been used in work with mumps virus

In veterinary work, Berkefeld filtrates of tissue infected with foot-and-mouth virus have been found to fix complement with immune sera (Lourens, 1909), unfiltered vesicle fluid has also been used (Mezincescu, Baroni, and Calinescu, 1923, Urbain, 1927).

2 Washed preparations of elementary bodies can be used, e g, in vaccinia, influenza or psittacosis

Preparations containing specific soluble (flocculable) substances can be used as sources of antigen, e g, in vaccinia, influenza, lymphocytic choriomeningitis, and mumps

3. Virus grown in tissue culture can form a source of antigen, e g, for vaccinia (Gilmore, 1931)

4 Virus grown in the egg yields a very suitable source of complement fixing antigen Thus, the chorio-allantoic membrane can be used with vaccinia virus. In psittacosis, lymphogranuloma, and other members of this group, antigen can be prepared from the yolk sac (see Section 7) The complement fixing antigen of lymphogranuloma can be enhanced by treatment with phenol, urea, ether, or boiling (see Chapter LXXII) Allantoic fluid can be used as a source of antigen, e.g in influenza (see Chapter LXII), or mumps (see Chapter XXIV). Amniotic fluid can also be used, e.g, in mumps

Irradiated whole embryo antigens have been used with equine encephalomyelitis

Yellow Fever

Hughes (1933) showed that the sera of monkeys which had recovered from the disease developed a precipitin capable of reacting with a precipitinogen occurring in the blood of monkeys during the acute phase of the disease. This precipitinogen, which was associated with the albumin fraction of the serum, was not the virus of yellow fever *per se*. The amount varied with the severity of the infection, and it was independent of the virus neutralizing antibody; it disappeared after the monkey recovered from the disease, and was thought to be responsible for stimulating the development of precipitating antibodies in the sera of animals after an attack of yellow fever.

A similar precipitin was found in convalescent human sera, which reacted with the precipitinogen occurring in the blood of infected monkeys

Serum derived from a monkey moribund from yellow fever was filtered through a Seitz filter, and antiserum obtained from a monkey which had recovered from a virulent (Asibi) strain of yellow fever was likewise treated The sera were then mixed in a test tube, incubated at 37° C. for 2 hours, and examined for the presence of cloudiness The ring technique was also tried by putting undiluted antiserum in the foot of a test tube (2 mm. in diameter) with the aid of a capillary pipette, and this was over-floated with serum derived from an ill animal. The two fluids were next incubated at 37° C. for 2 hours, and the results thereafter read in artificial light with the aid of a dark background A positive result was signified by the formation of a ring of turbidity at the fluid interface, while in the control test, in which normal monkey serum was substituted for antiserum, no such reaction occurred.

COMPLEMENT FIXATION IN THE STUDY OF VIRUSES

General Technique

The technique of performing a complement fixation test, using a virus suspension as antigen and its homologous antiserum, is little different from that of carrying out any routine complement fixation test (Mackie and McCartney, 1938, Browning and Mackie, 1937). The main difference lies in the fact that since viruses cannot be grown in large amounts on culture media like ordinary bacteria, antigen consisting of infected tissues must be employed. The use of the latter presents two disadvantages—the first is that it necessitates the use of an additional antigen and serum control. The second drawback is that since the concentration of virus in the tissues may be relatively low, its complement binding potency may be correspondingly feeble, so that a finely balanced test must be set up and sufficient time must be allowed for union of antigen and antibody to take place (Bedson, 1928)

The modes of preparing antigen have also received attention, different methods being used according to circumstances These are referred to below under their respective diseases The hemolytic system employed is similar to that used for the Wassermann test, and has usually consisted of a 3 or 5 per cent. suspension of either sheep or ox erythrocytes sensitized with about 5 m h d of homologous immune body Guinea-pig complement is universally employed, and this can be used either fresh or preserved by Sonnenschein's (1930) method (Mackie and McCartney, 1938), or else desiccated complement (Craigie, 1931) may be substituted It is well to use the mixed serum of several animals The minimum hemolytic dose of complement is determined in the usual way by titrating with sensitized corpuscles to be used for the particular experiment, and the test can be performed by the addition of a fixed volume of serum-antigen mixture to varying amounts of complement, or vice versa Thus, certain workers have preferred to use a fixed amount of complement in their tests, usually about 3 m h d , and add varying dilutions of serum, whereas others have chosen to employ a fixed volume of serum with

TABLE 10

VARIOLA-VACCINIA FIXATION TEST

Test

(*a*) Antigen, serial dilutions . 0 2 c c.
(*b*) Complement, 3 m h d in 0 2 c c 0 2 c c
(*c*) Serum, constant dilution . 0 1 c c

Controls

(1) Antigen, serial dilutions as in (*a*) 0 2 c c
Complement, 1 5 m h d in 0 2 c c 0 2 c c
Saline . . 0 1 c c
(2) Complement, 3 m h d in 0 2 c c (diluted 1 in 3) distributed as follows, the object of the arrangement being to ascertain whether complement had deteriorated during the long period of 16 to 18 hours' fixation at low temperature

Tube	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
Diluted comp 1 in 3	0.4	0 3	0 2	0 15	0 4	0 3	0 2	0 15	c c.
Saline . .	0 1	0 2	0 3	0 35		0 1	0 2	0 25	c c
Serum, constant dil as in (*c*)		.	.		0 1	0 1	0 1	0 1	c c
Estimated m h d of complement present	2 0	1 5	1 0	0 75	2 0	1 5	1 0	0 75	m h d.

[illegible]

Rift Valley Fever

Broom and Findlay (1932) have demonstrated the existence of specific complement fixing antibodies in the sera of humans and animals recovered from the disease. The antigen used was either a 2 per cent. saline extract prepared from fresh infected mouse or rat liver, or from similar tissues which had been desiccated over phosphorous pentoxide For the test, three different dilutions of the antigen suspension were used, namely, 0.25 c.c of 2 per cent., 1 per cent , and 0 5 per cent. [illegible] for a further 10 minutes and the results then read. The serum control consisted of normal human or animal serum, the antigen control being normal mouse or rat liver. In addition, an extra control was included in which a known positive serum was tested against infected liver tissue as well as normal liver.

Dengue

Partial success with complement fixation reactions has been claimed by Simmons, St. John, and Reynolds (1931) who at first tried to perform the test with antigen consisting of infected human blood, or the liver or spleen of infected *Macacus philippinensis* monkeys, but with negative results. Later, however, they were able to demonstrate fixation with antigen consisting of ground-up infected Aedes mosquitoes, for which purpose 279 insects were suspended in 95 per cent. alcohol, allowed to stand for 12 days, centrifuged, and the supernatant fluid diluted to make a 1 10 suspension in physiological saline

With such antigen, Simmons and his co-workers were able to show the presence of specific complement fixing antibodies in the sera of individuals who had suffered from dengue fever, the reaction being found positive from the first to the ninth day of the patient's illness

Yellow Fever

Frobisher (1929) and Davis (1931) have demonstrated that the sera of persons or monkeys after recovery from an attack of yellow fever develop specific comple-

It is reported that nonspecific fixation is lessened if sera are inactivated at 60° C for 15 minutes when using such antigens (Brown, 1947).

5. In work with the neurotropic agents (see Section II), brain tissue treated with ultraviolet light has been used as a potent yet noninfective source of complement fixing antigen. Antigens can also be prepared by freezing and thawing (see below).

6. Koktoantigen or boiled infected brain tissue was used as antigen for the complement fixation test by Torikata (1917), Nakagawa (1924), and Takaki, Bonis, and Koref (1926). Their method was as follows Brain tissue infected with a neurotropic virus preserved for 10 days or longer in 50 per cent. glycerin was ground up in 0 85 per cent. sodium chloride to yield a 10 per cent. suspension of brain tissue. This material was boiled for 30 minutes in a water bath, allowed to stand for 12 to 24 hours, sharply centrifuged, and the supernatant opalescent fluid used as antigen in the tests

Gildemeister and Heuer (1928) have criticized the findings of Takaki, Bonis, and Koref (1926) for, although the former agreed that complement fixation occurred between antivaccinial serum and homologous brain "koktoantigen," they questioned the specificity of the reaction as claimed by Takaki and his colleagues in respect of herpes virus Gildemeister and Heuer pointed out that normal brain tissue occasionally produced nonspecific complement fixation and, moreover, owing to the unreliability of such tests when performed on experimental animals, they expressed doubts concerning its practical value as a diagnostic reaction in man

The complement fixation test has also been performed in psittacosis with boiled antigen by Bedson (1937). Material was prepared as follows. A heavily infected mouse spleen was suspended in 5 0 c.c. of M/50 phosphate buffer solution of pH 7.6, and allowed to sediment for 24 hours in the refrigerator; the supernatant fluid was removed and centrifuged for 1½ hours in the angle centrifuge; the supernatant fluid was discarded, and the deposited material resuspended in 5.0 c c. of phosphate buffer, following which coarse fragments were removed by light centrifugation, the suspension was finally steamed for 30 minutes and employed as antigen in the test The control antigen consisted of normal mouse spleen

Boiled antigen prepared by this method was found useful for demonstrating the presence of complement fixing antibodies in the sera of human cases of psittacosis, and Bedson found that heating not only increased the sensitivity of the material, but also made it easier to preserve and safer to handle

In the case of herpes virus, however, heated antigen has given disappointing results, and Bedson and Bland (1929) reported that heating greatly diminished the antigenic activity of tissue extracts prepared from infected guinea-pig feet

7. Lyophilized benzene-extracted antigens prepared from brain or chick embryo show less tendency to give nonspecific reactions (Cox, 1948)

SOME REPRESENTATIVE COMPLEMENT FIXATION TESTS

Smallpox

Craigie and Wishart (1936) prepared antigen by macerating 40 mgm crusts in 0.2 c c 8 5 per cent. sodium chloride with a glass rod, 1 8 c c. of distilled water were added, and after centrifugation, the supernatant fluid was used as antigen The following scheme was recommended (see also p 370)

The complement employed for the reaction consists of guinea-pig serum which has been dried from the frozen state (see Craigie, 1931) Since prolonged cold room fixation is necessary, it will be observed that a special control is included, in order to ascertain whether the different dilutions of complement employed have deteriorated during the period of 16 to 18 hours' fixation

The serum employed is antivaccinial serum derived from rabbits immunized against vaccinia virus

day. Brains from all mice are emulsified in 10-fold the weight of diluent consisting of 2 per cent. normal guinea-pig serum (heated at 56° C. for 30 minutes) in normal saline, emulsification is performed in the Waring blender. The suspension is kept overnight in the refrigerator, and then centrifuged at 2,500 r.p.m for 30 minutes. The supernatant fluid is placed in 10 c.c. amounts in lusteroid tubes, and frozen and thawed by dipping alternately in CO_2 alcohol and water at 37° C This process is repeated until flocculation is noted, usually 4–5 times Finally, the extract is centrifuged *in an angle head machine at 7,000 r p m. for 1 hour; the supernatant con*stitutes the antigen after ad[illegible] used for as long as 4 months after [illegible] been prepared from the following [illegible] uelan encephalitis, Russian, louping-ill, Japanese B, West Nile, St. Louis, Theiler's GD VII strain, and lymphocytic choriomeningitis. These antigens are highly virulent Inactivation by [illegible] at 60° C. for [illegible] case of Venc[illegible] precipitate, ir[illegible] for at least 2 years

(2) The use of tissue extract as antigen and as an immunizing material for the production of antisera requires careful control For it is known that fresh normal *sera fix complement with a component in normal tissue extracts, which is sedi*mented at high speed, brain tissue injected in heterologous animals induces the formation of organ-specific antibodies. Accordingly, Casals immunizes animals with homologous tissue, and eliminates nonspecific fixation by centrifuging antigens at sufficiently high speed to sediment most of the normal tissue component responsible, or by inactivating the sera at 60–65° C. which destroys the normal serum component without affecting specific antibody

Animals are injected peritoneally on 2 occasions with 10 per cent formolized brain suspension, followed within 10 days by a peritoneal injection of live virus diluted 10^{-2}, repeated every 4–5 days. Within 4–6 weeks, most animals develop a high titer of antibodies Human sera which are positive in the Wassermann test give, as a rule, a nonspecific reaction with brain extracts This can be overcome by heating the sera at 65° C. instead of 60° C for 20 minutes.

(3) A preliminary titration of complement is made in saline, and two units are used in the test. Another titration is set up in the presence of antigen and saline solution.

(4) In the test, inactivated sera are diluted twofold (½, ¼, ⅛, 1/16 being usual), and 0 25 c c quantities are used. Then 0 25 c.c of antigen is added plus 2 units of complement in 0 5 c c Tubes are incubated at 2–4° C. for 18 hours The hemolytic system consists of 3 per cent sheep cells plus 3 units of rabbit antisheep hemolysin, and is added in 0 5 c c quantities The tubes are then incubated at 37° C. for 30 minutes

The same test can be applied in the case of an unknown antigen to be identified

(5) Casals describes certain additional safeguards Human blood should be drawn with aseptic precautions after fasting for several hours, and should be centrifuged after ½–1 hour at room temperature. Merthiolate may be added When sera cannot be tested shortly, they should be frozen They should be inactivated *at 60° C for 20 minutes, or at 65° if there is nonspecific fixation*

Importance of Adequate Controls

It cannot be too strongly emphasized that all serological tests such as complement fixation, agglutination, or neutralization tests, should be carefully controlled if the results are to be of value As alluded to earlier in this chapter, the subject demands particular attention in the study of human virus infections, since these agents are only cultivable along with living cells and the presence of the latter may

ment fixing antibodies. An antigen suitable for the test has been prepared by Frobisher (1931 *a*) according to the following procedure. The livers of several rhesus monkeys which had died from yellow fever were excised, freed from adventitious tissue, weighed, cut into small fragments with scissors, and ground in a mortar with sand to form a paste. This was then spread over the surface of a flat dish, placed in a desiccator over sulphuric acid, dried *in vacuo* for 18 to 24 hours, and the desiccate placed in hermetically sealed containers and stored in a refrigerator until required. Although saline suspensions of such material were found to act as an efficient antigen, better results were obtained using dried *liver tissue from which the fat had been removed. This was done by placing* the material in a Soxhlet apparatus and extracting the fat by treating it with ether for 8 hours or longer. The antigen was now prepared from the fat-free liver substance by first treating it with hypertonic salt solution and then diluting the mixture before use. To a measured bulk of tissue, about one-half of its weight of a 9 per cent solution of sodium chloride was added, allowed to macerate for 4 to 24 ... 0 09 per cent sodium chloride by addition of ... minutes, and the supernatant fluid passed through ... being used as antigen for the test.

Technique. Frobisher (1931) employed the following method. Sera to be tested were inactivated by heating at 56° C. for 30 minutes, 0.2 c.c. was placed in one test tube, and 0 1 c c. plus 0.1 c c of saline added to another, followed by the addition of 0 2 c.c. of diluted antigen to each tube. In a third tube 0 2 c c. of serum plus saline was placed. To all three tubes 2 m h d of guinea-pig complement were added The mixtures were then incubated at 37° C. for 1 hour, at the end of which 0 4 c c. of a hemolytic system consisting of 5 per cent sheep erythrocytes sensitized with 2 units of sheep antibody was added. The test was replaced in the incubator at 37° C. for a further period of 30 minutes, and the result read at the end of this time, or at such time as the controls showed complete lysis.

Psittacosis

The complement fixation reaction has been proved a valuable aid to the diagnosis of suspected cases of this disease (Bedson, 1935). The antigen employed for the reaction consists of a 5 per cent. saline suspension of virulent mouse spleen, *which has been allowed to stand in a refrigerator at +4° C for one week, in order* to annul any traces of natural complement (or alternatively, it is heated at 56° C for 30 minutes), and 0 1 c c bulk used for the test Boiled antigen was also shown to be effective by Bedson (1937) The patient's serum to be examined is made up 1 1 to 1 8 and 0 1 c c of each dilution employed Two m h d of complement are used throughout the test The mixtures are incubated at room temperature for 2 to 3 hours, 0 2 c c of sensitized cells added, the tubes placed in the water bath at 37° C. for 30 minutes, and the reaction read at the end of that time Bedson points out that the specific complement fixing power of these human psittacosis sera is of a low order and that a finely balanced test is essential to elicit the reaction Rigorous controls are also necessary to eliminate false reactions The reaction has been found to be positive as early as the twelfth day, and as late as the fifth week of the patient's illness

Neurotropic Infections

Casals (1947), who has had extensive experience of the complement fixation reaction in neurotropic virus infections, describes recent modifications in his technique

(1) To prepare antigens, 3- or 4-week old Swiss-W mice are inoculated cerebrally with 10^{-2} dilution of virus On the last day of the incubation period, the animals are placed in the icebox, and survivors are sacrificed on the following

this principle in order to enhance the amount of complement fixed in tests with antivaccinial sera, but the results were not satisfactory Later, Mackie and Finkelstein (1930) investigated the phenomenon of complement fixation produced by the interaction of normal serum with bacterial suspensions, and from their work they found that the normal sera of various mammals (e g, man, ox, sheep, horse, pig, white rat, rabbit, and guinea-pig) possessed the property of fixing complement with a wide variety of different bacteria.

These observations are of some interest because previously, Schultz, Bullock, and Lawrence (1928) suggested that positive complement fixation, using antivaccinial serum and vaccinial antigen prepared from infected rabbit skin, was in reality a nonspecific effect attributable to the existence of contaminant bacteria occurring in the antigen Schultz's allegations were subsequently reinvestigated by other workers, but received no support (Craigie and Tulloch, 1931).

In conclusion, we should like to add that, although specific complement fixing antibodies have been demonstrated in a great many virus diseases, these reactions are as a whole so delicate that the execution of such tests demands the utmost care and attention.

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per se cause certain nonspecific effects For example, although it is well known that many viruses function as efficient antigens in specific complement fixation reactions, yet earlier literature *on this same subject contained many contradictory* reports. In reference to this point, Parker and Muckenfuss (1933) have pointed out that some of the results reported were useless, either because they were insufficiently controlled, or else because they did not contain sufficient data For instance, when attempting to prepare a hyperimmune guinea-pig antivaccinal serum by inoculating the animal with vaccinia virus grown in rabbit testis, the experiment should be controlled by preparing another serum derived from a separate guinea-pig which has received a similar number of doses of normal rabbit testicu*lar extract The serum of a normal guinea-pig, if employed* as a *control* under such circumstances, would obviously be an inadequate check. This control is essential, [illegible] ment fixation, [illegible] inoculation [illegible] onsisting of normal healthy testis tissue should also be incorporated in the experiment in every case.

The state of our knowledge regarding the extent to which the parenteral injection of tissues derived from one species of animal into those of another gives rise to various immunological effects is as yet incompletely understood; for example, in the case of the Forssman reaction, the inoculation of a rabbit with the kidney tissue of a guinea-pig leads to the production of an hemolysin in the rabbit's serum for sheep erythrocytes. A hypothetical instance in which this phenomenon would interfere with the performance of a complement fixation test applied to a virus disease would be as follows if a rabbit was immunized with a virus contained in an infected guinea-pig's kidney, the rabbit's serum would not only develop complement fixing antibodies for the particular agent introduced, but would simultaneously produce an hemolysin for sheep cells Under such circumstances, an hemolytic system consisting of sensitized ox cells would have to be substituted for sheep cells. During the course of serological studies in connection with viruses, a careful watch should always be kept for the appearance of phenomena of a similar type, and no positive result should be accepted unless it has been proved beyond doubt that the effect is specifically due to the antigenic properties of the virus itself, and not one peculiar to the tissues of the animal in which the virus has been cultivated

In their carefully controlled experiments on influenza virus, Fairbrother and Hoyle (1937) have revealed the practical importance of this point, for, when they endeavored to apply the complement fixation test to ferret sera, they found from their control experiments that the complement fixation which occurred between anti-influenzal ferret serum and influenzal mouse lung was nonspecific in character, *as the same effect was obtained with normal mouse lung as antigen* Likewise the work of Gildemeister and Heuer (1928) showed that normal rabbit brain tissue could behave in a similar manner, and Hildebrand (1936) also proved that rabbit serum and normal human lymphatic glands could act in the same way. In the past ten years the researches of Bedson, Casals, Craigie, Fairbrother, and others, have improved the technique of complement fixation reactions applied to the study of viruses, and their results have indicated the great possibilities of such tests

The principal obstacle offered to the future development of this work, along lines which may be of practical utility in human diagnosis, still remains that of nonspecific complement fixation caused by tissue proteins As an academic problem the subject of nonspecific complement fixation is a complex one, and this has been intensively investigated *by Mackie and Finkelstein (1928) who have made a special* study of nonspecific complement fixation produced by the interaction of normal serum and certain nonantigenic substances Mackie and Finkelstein's work has also drawn attention to the possibility of increasing the amount of complement fixed by the addition of cholesterol to the antigen Gilmore (1931) endeavored to utilize

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In conclusion, we should like to add that, although specific complement fixing antibodies have been demonstrated in a great many virus diseases, these reactions are as a whole so delicate that the execution of such tests demands the utmost care and attention.

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CHAPTER XII

TISSUE CULTURE IN THE STUDY OF VIRUSES

Certain tissue cells will readily grow *in vitro* if adequately supported, nourished, and incubated, and such tissue cultures may be used in the study of viruses in various ways. Thus, they have been used in the study of virus inclusion bodies and life cycles, as a specialized culture medium to secure proliferation, and in the study of certain aspects of virus immunity. Before these applications can be considered, however, the methods used to secure growth of normal cells must first be discussed.

For fuller details than can be given here, a modern textbook on tissue culture should be consulted (e.g., Cameron, 1935; Parker, 1938).

Source of Cells for Tissue Culture

Epithelium is most readily obtained from the rabbit's cornea as follows: The [illegible] can be detached by slicing with the flat of the knife. These small pieces are placed in a covered bath of Tyrode's solution, and incubated at 37° C. until required.

Fibroblasts can be readily obtained by culturing small pieces of such parenchymatous organs as the lung, liver, kidney, or heart. We have obtained excellent growths of fibroblasts from mouse lungs.

Testis. Minced rabbit testis is frequently employed; mouse testis may also be used.

Chick embryo cells. Epithelium may be obtained from the lung, and fibroblasts from the leg muscles, of the chick embryo. The whole embryo is frequently used in media of the Maitland type.

Mouse and rat embryos may be used. They are removed 2 to 3 days before term and finely minced.

Materials required for Tissue Culture

Slides measure 3 by 1½ in. with a central hollow of 1 in. diameter, and should be wrapped in Kraft paper.

Coverslips should be of thickness number 1 and contained in a small tin or glass jar.

Pasteur pipettes have a very fine point and are contained in a tin.

Watch-glasses are wrapped singly in Kraft paper.

Instruments: a selection of seekers, forceps, and probes is contained in a tin box.

All the above are sterilized in the hot-air oven.

Glass tubes measure 3 in. long by ½ in. in diameter, they are corked at either end by a rubber bung, and should be sterilized by autoclaving.

Incubator: this should be set at 37° C., contain numerous rigid trays, and have a moist atmosphere.

Tyrode's solution is prepared as follows: sodium chloride, 8 gm.; potassium chloride, 0.2 gm., dextrose, 1 gm., acid sodium phosphate, 0.05 gm., sodium carbonate, 1 gm., magnesium chloride (5 per cent. anhydrous salt), 4 c.c., calcium chloride (5 per cent. anhydrous salt), 4 c.c., distilled water, 1,000 c.c.

This strongly alkaline solution is adjusted to pH 7.4 by addition of a 5 per cent. solution of 85 per cent. phosphoric acid, approximately 1 to 1.5 c.c. being required

per 1,000 c.c. of solution. Acidity should be corrected by addition of 10 per cent. sodium carbonate solution. The solution is finally sterilized by filtration through a single disk of a Seitz (EK) filter at 30 mm of mercury, it is then distributed aseptically into small bottles and tubes, and stored in the ice chest until required.

Sealing mixture consists of vaseline, 7 parts, and paraffin (m.p 54° C.), 1 part, it is sterilized by placing a small screw-topped bottleful in boiling water, or on a hot plate

Nutrient fluids: plasma. Chicken plasma is recommended, and is obtained by bleeding a young bird which has been starved for 24 hours The wing is carefully plucked and sterilized, and the vessels in close relation to the humerus are located Two ligatures about ⅓ in. apart are then passed loosely round the vessels, which are cut in between these points As the blood flows out, it is received into a series of test tubes containing heparin to prevent clotting. After sufficient blood has been collected, the ligatures are tightened and bleeding arrested. The bird should receive a large dose of saline solution given subcutaneously to make up for the loss of fluid. Rabbit plasma may also be used, the heparinized blood being centrifuged, and the plasma separated.

It has been found that mammalian plasma can be stored for a long time in the ice-chest, if treated in the following way (Baroni, 1930, and personal communication) Whole blood is mixed with Tyrode's solution (10 to 15 times concentrated), and then centrifuged The plasma is separated and stored. When serum is required for use, 1 part of plasma is mixed with 3 or 4 25 parts of distilled water, when a clot forms Plasma (and embryo extract) may be frozen and dried *in vacuo* (Hetherington and Craig, 1939).

Rabbit serum. A rabbit is heart-punctured, and the blood allowed to clot, the serum being separated after "ringing" and centrifugation. Serum can be stored in the lyophil form (Hetherington, 1944).

Spleen extract. A freshly removed rabbit's spleen is ground up with approximately 10 parts of Tyrode's solution. After low-speed centrifugation for ¼ hour the clear supernatant is removed from the deposit, and constitutes spleen extract, which may be stored in the ice chest if so desired. We recommend this extract, although embryo extract can also be used.

Embryo extract. The egg shell of an 8- to 10-day chick embryo is carefully disinfected with spirit and iodine, and when dry is partially opened with a dental drill The embryo is removed, then minced and ground-up with 3 c c. of Tyrode's solution. After slow centrifugation for a few minutes, the supernatant fluid is withdrawn to constitute embryo extract

METHODS OF TISSUE CULTURE FOR THE STUDY OF VIRUS INCLUSIONS

1. The Clotted Plasma Method

(a) Preparation.

For speed and accuracy the following routine is recommended

1 Three working places are desirable, the workers sitting side by side

2 The worker on the left is responsible for unwrapping sterile hollow-ground slides, *ringing round* the hollow with sealing mixture (kept melted by a water bath or electric hot-plate), and passing them on to the central worker

3. This worker mixes up the nutrient fluids in the following proportions chick plasma, 2 parts, rabbit serum, 2 parts, and spleen extract, 1 part, this is carried out by pipetting the correct number of drops of each material into one of the sterile watch-glasses The contents are mixed by sucking up once or twice in another sterile Pasteur pipette

4 Meanwhile, the worker on the right, working with his hands inside a cabinet,

is placing 2 to 3 squares of cornea, or other tissue, onto sterile coverslips These pieces of tissue are referred to as "explants"

5 The central worker, working through the side door of the box, covers each piece of tissue with a drop of nutrient fluid, and then picks up each slip with a vaselined slide. These are left upside down for a few minutes, until the plasma clots, when they are inverted and placed on trays in the incubator.

(*b*) **Progress.**

Cultures may be examined microscopically after 36 to 48 hours, when, if successful, a considerable growth should be apparent Epithelium spreads out as a continuous sheet around the explant, and has a very regular growing edge. Each cell is roughly polyhedral and rather granular, by appropriate methods much of this granularity can be shown to be due to fat. Fibroblasts grow out more in the form of individual strands and filaments, thus giving the edge of the culture an irregular appearance Cells growing
growing vertically appear
to the explant will probal
of no consequence as pure cultures are not essential in virus work

Cultures may be infected at the time of setting up, or later. Bland and Robinow (1939) infected their cultures after about 3 days' growth, when the culture medium had partly liquefied In these experiments, the cultures were washed with saline to remove excess virus inoculum, and then nutrient fluid was added and the growths remounted.

(*c*) **Subcultivation.**

Growth should occur for 5 to 7 days, before the nutrient fluid is exhausted, the results of most virus experiments should be evident by then, but occasionally it may be necessary to subculture If so, the slip is removed from the slide, and placed, culture upward, on a dark background. The clot is then cut through the center with a sterile razor blade, down to the glass The old clot is picked off, and the two culture fragments are floated out in a warm bath of Tyrode's solution Two fresh cultures can then be prepared by covering these pieces with nutrient fluid on a coverslip, as already described.

(*d*) **Histological examination.**

Cultures embedded in a solid plasma clot are difficult to stain, as fixation frequently results in the formation of an insoluble precipitate The following method, which dispenses with previous fixation, can be recommended

1 HEMATEIN AND EOSIN METHOD (see Rhodes, 1937).

Solutions required

Hematein methylated spirit, 75 c c , glacial acetic acid, 5 c c., formalin, 10 c c , Mayer's acid hemalum, 5 c c.

Alcoholic eosin saturated solutions of alcoholic eosin and methylated spirit, equal parts.

Procedure

1 Remove slip, float, culture down, in a bath of hematein for 10 minutes
2 Wash in methylated spirit and then in tap water
3 Dehydrate partially with methylated spirit and absolute alcohol.
4 Counterstain in alcoholic eosin for 1 minute.
5 Wash with absolute alcohol, and leave in xylol till dehydration is complete. Mount in balsam.

This method stains nuclei blue and cytoplasm pink A number of other methods may also be used, most of these involve preliminary fixation

2. PRELIMINARY TREATMENT WITH WARM FLUID

It is said that if the slip is floated, culture downward, in a bath of warm Ringer's or Tyrode's solution for approximately 30 minutes, the plasma becomes detached, and even if not, subsequent fixation and staining will be rendered easier.

3. FIXATION METHODS.

Fix the culture, after the above treatment, in iodine vapor, formalin, or osmic acid Another method is to fill up the hollow of the slide with 2 per cent. formalin in saline, and then float the slip, culture downward, on the surface.

4 DEHYDRATION AND EMBEDDING.

Thereafter, the culture may be sliced off the slip with a sharp razor blade, dehydrated, cleared, and embedded in paraffin Sections are cut parallel to the axis of the slip and may be stained by any suitable method

5 FISCHER'S METHODS (see 1925).

Method (a). Procedure.

1. Fix in 2 per cent formalin in Ringer's solution for 1 hour.
2. Wash in running water for 3 hours, and distilled water for 1 hour
3. Place in 10 per cent. aqueous hematoxylin for 10 to 12 hours, wash for ½ hour.
4. Treat with 50, 75, 95 per cent. alcohols for 5 minutes each.
5. Place for 10 minutes in each of the following mixtures: 95 per cent. acetone and 5 per cent. xylol, 70 per cent. acetone and 30 per cent xylol, and 30 per cent acetone and 70 per cent. xylol
6. Clear in xylol, and mount in balsam

Method (b) Solution required.

Saturated alcoholic solution of methylene blue, 30 c.c., 1/10,000 potassium hydroxide, 100 c c, ripen in incubator for 1 month

Procedure

1. Fix and wash as above
2. Apply distilled water for 5 minutes.
3. Pour on methylene blue, heat till steam rises, leave for 15 minutes, wash
4. Dehydrate and clear as above, except that for alcohols 1 minute is enough, and for acetone-xylol mixtures 2 minutes each.

6 STRANGEWAYS'S METHOD (see 1924).

Solutions required.

Acetic alcohol 0 5 per cent glacial acetic acid in 70 per cent. alcohol
Iron alum 2 5 gm iron alum in 100 c c distilled water

Procedure.

1. After treating in warm fluid, as above, place slip in a watch-glass of acetic alcohol for 5 minutes, wash in running distilled water for 5 minutes
2. Apply iron alum for 30 minutes, wash as in 1
3. Float slip, culture down, on the surface of ½ per cent. watery hematoxylin for 5 minutes at 37° C, wash for 2 to 3 minutes.
4. Differentiate in iron alum, control microscopically, wash as in 1.
5. Mount in Farrant's mountant.

7. Stain for Fat (Strangeways, 1924).

Procedure.

1. Proceed as in above method, but after differentiation in alum and washing, place in saturated solution of either Scharlach R or Sudan III for 5 minutes, wash in water.
2. Mount in Farrant's mountant

8 Bland and Robinow's (1939) Method

Procedure.

1. Float cultures (on glass mounts) for a minute on warm saline, and then expose to a strong concentration of osmic acid for 5 to 6 minutes.
2. Harden in 70 per cent. alcohol for 5 minutes, wash in distilled water.
3. Stain overnight in Hollborn's Giemsa (20 drops to 15 c.c. buffered distilled water, pH 6 9).
4. Differentiate in acetone-xylol mixtures, before this, weak acetic acid should be applied to correct over-red preparations.

2. Miyagawa's Method (Miyagawa *et al*, 1936)

This is a variation of the usual clotted plasma method, and has been used to study the development of the granulocorpuscles of lymphogranuloma inguinale.

(*a*) *Preparation.*

Guinea-pig plasma (heparinized) and mouse spleen extract are mixed in the hollow of a hollow-ground slide, this forms a thin film, which soon clots Fragments of normal mouse testicle or spleen are embedded in the clot, alongside fragments of a mouse brain infected with LGI The whole is then covered with more spleen extract, sealed, and covered with a mica slip.

(*b*) *Progress.*

New cells can be observed to grow out of the normal fragment, which gradually becomes infected with virus from the neighboring brain tissue.

(*c*) *Subcultivation*

This is carried out by removing the liquid extract and infected brain tissue, washing the testicle or spleen fragment in Tyrode's solution, and embedding it in another plasma clot Some healthy testicle or spleen is now implanted beside this infected fragment, and the whole covered and sealed as before

(*d*) *Histological examination*

The liquid extract is removed, the tissue fragments are then fixed in formalin, and stuck to an albumenized slide Giemsa's stain shows typical granulocorpuscles in the newly formed cells at the edge of the growth.

3. The Liquid Culture Method

(*a*) *Preparation*

These cultures are set up in the same way as in the clotted plasma method, except that the nutrient fluid varies. Thus, the cells are grown in mixtures of serum and Tyrode's solution, or plasma and serum, without added extract Generally speaking, the growth of cells obtained by this method is poor. The method must, however, be used for micromanipulation studies, as the needles cannot work through clotted plasma

(b) Progress.

Progress is followed microscopically, great care being taken not to shake or jolt the culture.

(c) Subcultivation

The exhausted nutrient fluid is simply sucked off, and replaced with a fresh supply.

(d) Histological examination.

To those methods already mentioned in previous sections may be added one other:

GIEMSA'S METHOD (Bland and Canti, 1935).

Buffer solution required: potassium dihydrogen phosphate, 1 gm; disodium hydrogen phosphate, 2 gm, distilled water, 1,000 c.c.
Stain: 2 drops of Giemsa in 1 c.c. of buffer solution.

Procedure

1. Gently wash culture in saline, and fix in methyl alcohol for 5 minutes.
2. Rinse in buffer solution, remove, and discard the center of the explant.
3. Stain for 24 hours at room temperature.
4. Differentiate in acetone, clear in xylol, and mount in balsam.

4. The Tube Culture Method

This method has been used in studies on vaccinial inclusion bodies (Rivers *et al*, 1928–9, 1929 *b*; Rhodes and van Rooyen, 1937).

(a) Preparation.

A rabbit's eye is freed in the orbital cavity, the cornea is washed with saline, and dissected out entire. It is then cut up in a bath of Tyrode's solution into 8 to 12 squares of approximately equal size. Nutrient fluid, as used in the clotted plasma method, is then pipetted into each of a number of glass tubes to a depth of ½ in, these tubes are closed at either end by a bung When the clot has formed, a piece of cornea is placed horizontally on top, and covered with another layer of fluid. Soon the contents of the tube have formed into a solid clot, in the center of which lies the piece of cornea

(b) Progress

This can only be estimated by examining histological preparations.

(c) Subcultivation

This cannot be carried out, but the tissue should grow for at least 7 days before the nutrient fluid is exhausted

(d) Histological examination

1 Remove both stoppers, gently tap the tube, and the entire clot should slip out Fix in Helly's or other fixative for a short time

2 Thereafter, dehydration, clearing, and embedding are carried out as usual Paraffin sections are cut parallel to the sides of the tube, thus giving, in the center of the clot, a radial section of cornea

3. Sections may be stained by any desired method, e.g, Mann's stain

This method has an advantage over the slide methods, for a perfect histological preparation is obtainable Such sections show that cellular proliferation has occurred in the depths of the cornea, as well as in the superficial epithelium Thus,

the fibroblasts in the substantia propria increase in numbers, and there is considerable thickening of the epithelial layers

5. Carrel's (1923) Method

(*a*) *Preparation*

Cultures are contained in special Carrel flasks, of which various shapes have been designed. The commonest shape for bacteriological purposes is that known as Type D. The diameter of these flasks may be either 5 or 8 cm. The other shapes are. Type A, resembling D in shape, but fitted with a top opening, 3 cm. in diameter, Type B, resembling D, but fitted with two side arms, Type C has a longer neck than D, and top and bottom openings; Type E resembles D, but has

plasma, which is run uniformly

with 5 per cent. tissue extract

added) are then mixed with the plasma The fragments of tissue to be cultured are embedded in the plasma just before it clots, by using a long narrow metal spatula When the clot forms, some fluid medium is run over the surface; if the cells are epithelial or fibroblastic, use 25 per cent. tissue extract in Tyrode, and if leukocytic, use 2 parts Tyrode and 1 part each of serum and tissue extract (Cameron, 1935).

(*b*) *Progress and* (*c*) *Subcultivation.*

The fluid requires to be changed every 2 to 5 days, to do this, carefully flame the neck of the flask, suck out the old fluid, and add fresh. When the original clot becomes exhausted a section is cut out with a spatula, the tissue dissected out and re-embedded in a new flask.

(*d*) *Histological examination.*

Sections of clot may be removed with a spatula, fixed and taken through to paraffin, excellent results being obtained after suitable staining In other cases, fixative may be poured into the flask, and the fixed tissue removed by breaking the glass.

THE STUDY OF VIRUS INCLUSION BODIES AND LIFE CYCLES

Various viruses produce inclusions when added to, or incorporated in, tissue cultures of the above types. Thus, with slide methods, lymphogranuloma inguinale (see p 775), vaccinia (see p. 344), and psittacosis (see p. 750), have been shown to form inclusions. The virus of herpes has been studied by the tube culture method and inclusion bodies described (Rivers *et al*, 1928–9, 1929 *b*). Rhodes and van Rooyen (1937), employing the same method in the study of vaccinia, found that inclusions developed Carrel's method has been used by Andrewes to demonstrate inclusion bodies in virus III of rabbits (1929 *a*, *b*), and herpes (1930)

The following method was used by Bland and Canti (1935) in their study of the life cycle of psittacosis

1. The nutrient fluid was prepared as follows 2 parts of chick plasma were mixed with 3 parts of chick embryo extract (2 5 c c. saline to each minced to days' embryo), when clotting occurred, fluid was expressed. This fluid was diluted with an equal volume of saline, and centrifuged at high speed for 1 hour, to remove refractile granules Epithelial cells were cultured directly in this fluid, but fibroblasts were first grown by the clotted plasma method, and subcultured into this later

2 Explants of the muscle or lung tissue to be cultured were mounted by Maxi-

ter) being fixed

a small amount

3. Cultures were infected by adding virus to the nutrient fluid at the onset, or

after a certain amount of growth had occurred. In the latter case, the medium was replaced by a drop of virus, and the culture incubated for 1 hour. Then the culture was re-opened, the virus washed off, and fresh nutrient fluid added.

4. Such infected preparations were examined after a further period of growth, either by Giemsa's stain, or by darkground microscopy. For the latter examination cultures were mounted as contact specimens The thick center of the culture was discarded and the slips were then inverted over a thin slide to which thin strips of coverslip were luted with distilled water.

In this study the various developmental phases in the life history of the psittacosis virus were worked out. Somewhat similar studies have been made with vaccinia (Bland and Robinow, 1939).

Merling (1945) has carried out prolonged observation on the phagocytosis of vaccinia virus by leukocytes, using fragments of cornea in Tyrode's solution on slides. Preparations were stained by Herzberg's Victoria blue 4R and examined by transmitted and darkground illumination.

Chick embryo cells grown in tissue culture can be examined with the electron microscope (Porter, Claude, and Fulham, 1945).

THE PROPAGATION OF VIRUSES

Ever since viruses were first discovered, at the close of last century, attempts have been made to culture them. Although viruses resemble bacteria in many respects, they differ radically in that they cannot be cultivated on ordinary laboratory media, however much enriched. The whole trend of modern work shows that viruses will only grow in the presence of living cells, and it has been suggested that they exhibit the most extreme degree of parasitism in nature.

Prior to 1928, a number of investigators had obtained growths of vaccinia virus in tissue culture, the usual method was to use hanging-drop preparations of embryo tissue or testis suspended in serum or plasma, with virus added. This technique was somewhat uncertain, it did not permit of large-scale operations, nor of ease and accuracy of titration. A real advance was initiated when Maitland and Maitland (1928) grew vaccinia virus in a medium containing hen's kidney and serum.

Maitland's Media

Originally, the inoculum consisted of the testis of a rabbit inoculated 4 days before with vaccine lymph, ground in sand, with M/50 phosphate (pH 7.6) added After centrifuging, the supernatant fluid (approximately 5 c.c.) was used for the primary inoculum

The kidneys of a hen were cut up with scissors and 0.66 gm. placed in a flask, 1.33 c.c. of virus inoculum (diluted 1/66 with Tyrode's solution) being added After standing in the cold room for 4 hours, 12 c.c. Tyrode and 6 c.c. hen serum were added, thus making a final dilution of virus inoculum of 1/100 Thereafter, 2 c.c. amounts were distributed into Carrel flasks (Type D), and incubated aerobically at 37° C, without caps Cultures were titrated for the content of virus by grinding with sand, centrifuging, and injecting 0.2 c.c. of dilutions of the supernatant intradermally in rabbits During four successive subcultures, virus increased approximately 25×10^6 times In these cultures it was not possible to detect any tissue growth, after 24 hours the smallest pieces of kidney had begun to disintegrate, this process of autolysis being complete by the third day.

Maitland and Laing (1930) substituted rabbit for hen tissues, they placed 0.33 c.c. of freshly minced kidney or testis into a wide tube. Vaccinial material was then added and gently mixed, 3 c.c. of serum, and enough Tyrode's solution to give a final bulk of 10 c.c., were then added (serum last). The mixture was finally distributed into Carrel flasks in 2 c.c. amounts Vaccinia would not multiply in liver or spleen cultures.

Maitland, Laing, and Lyth (1932) found that vaccinia would grow readily in minced chick embryo and Tyrode's solution without serum.

In Maitland's most recent publication, the following method is recommended (Maitland and Laing, 1941): Minced adult rabbit kidney, 0.35–0.4 gm., is added to 10 c.c. of Tyrode's solution in a small conical flask, 0.1 c.c. of virus suspension (e.g., the supernatant from a previous culture) is added. The contents are thoroughly mixed, and 2.5 c.c. distributed into Carrel flasks. Two flasks should be incubated for 7 days at 37° C. in a moist atmosphere, and then titrated. One flask is prepared for titration at once, to assess the virus titer before incubation.

To titrate cultures, the contents of flasks are decanted into a sterile mortar, sterile abrasive is added, and the contents thoroughly ground. Fivefold dilutions of the supernatant fluid (after centrifugation) are made in sterile saline and 0.2 from each c.c. is inoculated intradermally in the rabbit's back.

Other Media of Similar Type

1. *Findlay's (1928) method.* In this medium fowl-pox virus was added to the skin and brain of a 12- to 15-day chick embryo, after standing in the ice chest for 2 days, equal amounts of embryo-virus suspension were distributed into flasks containing 5 c.c. chick plasma, and incubated at 37° C. for 4 days.

2. *Smith's (1935) method.* Minced 8- to 10-day chick embryos were introduced into Carrel flasks in amounts from 0.05 c.c. to 0.1 c.c. The virus (influenza) inoculum was dropped onto the tissue, it was left for 10 minutes, and then 2 c.c. of Tyrode's solution were added. The flask was rotated to distribute its contents, and incubated at 37° C. Subcultivation was carried out by transferring 0.05 c.c. of fluid from flask to flask.

3. *Tamura's methods* (1934, 1935, D'Aunoy *et al.*, 1935). A small quantity of virus-containing material was added to 10 c.c. of Tyrode's solution, in which was either a piece of guinea-pig kidney or liver, or rabbit kidney.

4. *Dochez, Mills, and Kneeland's (1936) method.* Minced 10-day chick embryo was pipetted into two culture tubes (2 cm. diameter) in equal amounts, 10 c.c. of special peptone broth were then added (sodium chloride free, made with nontoxic casein peptone, and containing 0.1 per cent gelatin). Sufficient cysteine hydrochloride was then added to give a concentration of 1/2,000 (cysteine was prepared in 1 per cent solution, neutralized with caustic soda, autoclaved, and kept under a vaseline seal). The culture tubes were sealed with vaseline, and stored at 4° C. till required. Cultures were initiated by introducing virus-containing material (common cold) through the vaseline seal. Subcultures were carried out by transferring 1 c.c. of fluid to a fresh culture tube, at intervals of from 2 to 9 days.

5. *Li and Rivers's (1930) method* (see also Rivers and Ward, 1933 *c*). This method was introduced to grow vaccinia virus on a large scale. The medium consisted of rabbit testis, rabbit serum, and Tyrode's solution for primary growths, after subculturing, chick embryo and Tyrode's solution were substituted. The conical flask made to contain this medium was of an unusual shape, being fitted with a side ventilating arm and a gutter neck to catch condensation water. The flasks were made in two sizes to hold 17 c.c. and 6 c.c., respectively.

6. *Webster and Clow's (1936, 1937) method.* To grow rabies virus in bulk, these authors used a mixture of Tyrode's solution (9 parts) and monkey or horse serum (1 part), 4 c.c. were placed in a 50 c.c. Erlenmeyer flask, and mouse, rabbit, or chick brain added. Rat or mouse embryo, with chick plasma, may also be used (Bernkopf and Kligler, 1937).

The Roller Tube Technique

Of recent years, this technique has been adopted to secure growth of viruses by various workers: vaccinia (Feller, Enders, and Weller, 1940), influenza (Pearson and Enders, 1941), and psittacosis (Morgan and Wiseman, 1946).

Tissue fragments are embedded in plasma which is distributed over the wall of a test tube. Nutrient fluid is added, and the tube rotated in a horizontal position at 37° C.

The following method is that described by Feller, Enders, and Weller for the growth of vaccinia.

Tissue. Minced whole chick embryos (minus the eyes) of 8–10 days' development, or minced hearts from 10–12 day embryos are employed.

Embryonic extract. Minced 10–12 day chick embryos (minus eyes) are extracted with an equal volume of Simms' solution for 20 minutes at 37° C. After centrifugation for 20 minutes at 2,400 r.p.m., the supernatant fluid is carefully removed and stored in the ice-box till required (For formula of Simms' solution, see below.)

Plasma and serum are obtained from adult chickens by heart puncture, and heparinizing (for plasma) or defibrinating (for serum).

Assembling cultures Clean 20×150 mm. pyrex test tubes are used, and 5 drops of plasma spread over the lower ⅔ of the surface. Fragments of minced tissue (30–50) are then added. The plasma clots, and any excess unclotted fluid can be removed. Nutrient fluid 1.6 c.c. is then added (Simms' solution, 7 parts, embryo extract, 2 parts, chicken serum, 1 part). The tube is then rocked to wet the whole surface of the tissue and plasma. The tube is sealed with a rubber stopper fitted with a short piece of pyrex tubing closed with a rubber cap.

Incubation is carried out horizontally in a rotating device turning 8–10 times an hour.

Routine attention. Each day the nutrient fluid is removed by passing a Pasteur pipette through the small pyrex tube About 20 c.c of air which has been drawn through sterile cotton-wool is injected with a syringe. Nutrient fluid, 1 6 c.c., is added, and the tube sealed and returned to the rotator. Every 5 days or so the tube is drained of fluid, and more plasma quickly distributed over the cells. Clotting is accelerated by adding a few drops of embryo extract.

Addition of virus. Virus suspension is diluted with Simms' solution, and incorporated in the nutrient fluid. The inoculum is removed from the cultures after 24 hours

Titration The tissue and plasma coagulum is scraped off and ground in a mortar with broth. Serial dilutions are made and injected in animals as indicated

Mounting is carried out by including coverslips in the tubes used for cultures

Staining The coverslips are removed and stained, or else the cultures can be fixed *in situ* in the tube, dehydrated, and the coagulum and cells scraped off later

In work with psittacosis, Morgan and Wiseman (1946) found that growth occurred equally well in: (1) a mixture of Simms' solution, 21 parts, 50 per cent chick embryo extract, 3 parts, and rabbit serum, 3 parts, or (2) Simms' solution, 2 parts, serum ultrafiltrate, 1 part The second fluid had the advantage of simplicity and freedom from extraneous tissue. The cultures were grown for 48 hours prior to inoculation Good growths occurred, and mice were protected with formolized vaccines

Solid Media

Yanamura and Meyer (1941) used a modified Zinsser solid medium for growing psittacosis:

Part 1 Distilled water, 540 c.c , Simms' solution A, concentrated, 30 c.c., agar, 16 gm.

Part 2 Horse serum, 200 c.c , Simms' solution B, concentrated, 30 c.c.

Part 1 is autoclaved at 15 lbs pressure for 30 minutes, and cooled to 45° C Part 2 is filtered through a Berkefeld candle, and warmed to 45° C The two parts are mixed, distributed in 10 c.c amounts in 20×150 m.m. test tubes, and sloped For mass cultures, 100 c.c. amounts are distributed in 200 c.c. Kolle flasks.

For routine cultures, 0.25 c.c. fresh chick embryo pulp (as dry as possible) is introduced into each slant, and infected with virus inoculum held on a platinum loop.

For mass cultures, about 3.5 c.c. of chick pulp are distributed in test tubes and mixed with the infected tissues. The infected pulp is then spread evenly on the surface of the Kolle flasks.

Tubes and flasks are sealed with paraffin over cotton wool plugs, and incubated for 3–4 days at 37° C.

Media Incorporating Serum Ultrafiltrate

A preparation known as serum ultrafiltrate has been widely used by Simms, Sanders, and others for the cultivation of viruses. The paper by Simms and Sanders (1942) gives full technical details In the preparation and maintenance of ultrafiltrate cultures for virus propagation 4 points are of special importance. The volume of tissue should not exceed 1 part in 100 of medium. The pH should be maintained at 7.2–7.6. The pH should be adjusted with CO_2 as the tissues need bicarbonate. To prevent escape of CO_2 the flasks should be plugged with rubber and not wool stoppers. Ultrafiltrate can be used in small flasks, or large 500 c.c. Erlenmeyer flasks.

Preparation of ultrafiltrate. Serum ultrafiltrate is produced from ox serum by filtration through collodion in a specially designed apparatus. The ultrafiltrate is water-clear and free from protein. Phenol red is added in suitable concentration. The ultrafiltrate is then adjusted to pH 7.0–7.2 with CO_2, sterilized by filtration through a sintered glass filter, stoppered, and stored in the icebox. For use, ultrafiltrate is diluted 1 part to 2 parts of salt solution (this dilution is known as UF/3).

Simm's physiological solutions. Various solutions are used, X7 and X6 being prepared as follows:

	20 times concentrated gm./liter	*Final composition of X7 gm./liter*
Mother solution 1		
NaCl	160	8
KCl	4.0	0.20
$CaCl_2 \cdot 2H_2O$	2.94	0.147
$MgCl_2 \cdot 6H_2O$	4.06	c.203
Mother solution 2		
$NaHCO_3$	20.2	1.01
Na_2HPO_4	4.26	0.213
Dextrose	20.0	1.00
Phenol red	0.2	0.01

X6 solution differs from X7 in the phenol red concentration being 5 times that of X7 Mother solutions 1 and 2 are prepared in 20-fold concentrations as shown in the table, and stored separately. For use, 50 c.c. of solution 1 is diluted with 900 c.c. of freshly redistilled water and autoclaved, when 50 c.c. of solution 2 are added

Another solution (Z) contains no bicarbonate, and does not change pH on exposure to air It can be used for temporary bathing or storage of tissues for short periods Z is prepared by substituting mother solution 3 for solution 2.

	20 times concentrated gm./liter	*Final composition of Z solution gm./liter*
Mother solution 3		
$NaH_2PO_4 \cdot H_2O$	0.42	0.021
Na_2HPO_4	3.8	0.190
Dextrose	20.0	1.00

Propagation of viruses in ultrafiltrate cultures. The procedure is as follows

1. The embryo organs are removed aseptically and washed twice in Z solution Tissue is then minced with scissors in fresh ultrafiltrate (diluted with Z solution) into pieces not exceeding 2 mm. The minced tissue is again washed, and then suspended in ultrafiltrate in Z solution.
2. Into each of a number of 50 c.c. Erlenmeyer flasks are placed 8 c.c. of dilute ultrafiltrate (1 part UF—2 parts X7), and each flask is stoppered with a rubber bung.
3. The flasks are opened individually and 0.06 c c. of tissue suspension is added
4. *Before closing, 5 per cent. CO_2 in air is introduced aseptically.*
5. The cultures are incubated at 37° C. for 24 hours, and are then inoculated with 0.1 c.c. of material containing active virus.

Field cultures. Sanders and Huang (1944) found that small amounts of embryonic tissues would survive in 2 c.c. of serum ultrafiltrate diluted with 2 parts of Simms' physiological solution for the following times: at 4–6° C. for 3 weeks, at 25° C. for 35 days; and at 37° C. for at least 4 weeks. These could be used as simple tissue cultures under field conditions.

The importance of pH. Huang (1943) found that in noninfected tissue cultures, the pH shifted to the acid side, and in the presence of phenol red the color changed from red to yellow within 2 hours at 37.5° C. in a partial vacuum. When tissue was heavily infected with virus such as Western equine encephalomyelitis or St. Louis, there was either very little or no change in the pH.

Cultivation of Neurotropic Viruses

Many of these agents have been grown in media consisting essentially of 10 per cent. serum-Tyrode with small portions of the following type of tissue (minced)· mouse embryo brain, whole mouse embryo, whole chick embryo, and chick embryo without nervous system

By growth in chick embryo without nervous system, Koprowski and Lennette (1946) were able to alter the neurotropic properties of West Nile virus, so that it became less virulent for mice by extraneural routes.

Attempts to grow Vaccinia in Cell-free Medium

The comparative ease with which vaccinia virus grew in Maitland's and similar media led Eagles and his associates to attempt its cultivation in cell-free media. Various series of these experiments were carried out

1 Eagles and McClean (1930, 1931) prepared a kidney extract as follows a rabbit kidney was removed aseptically and very finely minced in 3 to 4 c c of Tyrode's solution After high-speed centrifugation for 20 minutes the supernatant fluid constituted kidney extract, this was examined for cells, but none could be demonstrated The medium, designated "cell-free," was prepared from this kidney extract, vaccinia virus, and Tyrode's solution, with or without the addition of rabbit's serum, incubation was carried out in Carrel flasks. Vaccinia virus increased *considerably in amount in these cultures, e g, in one case thirteen subcultures* showed an approximate virus increase of 10^{43}, although the approximate dilution of the original seeding of virus was 10^{-22}.

2. Eagles and Kordi (1932) prepared another type of extract A rabbit kidney was chopped and ground up in 2 c c of 9 per cent (hypertonic) saline, the suspension being divided into two equal portions in centrifuge tubes One tube was left at room temperature for at least 1 hour, while the other was frozen at −12° C to −13° C. for 1 hour. The frozen tube was then thawed in water (80° C) By this means it was hoped to carry further the cellular disintegration begun by hypertonic saline. *Sterile distilled water was then added to each tube,* to make the concentration of saline normal, and centrifugation at 4,000 r.p.m. carried out for 1 hour

The supernatant fluid was likewise centrifuged, when stained films were examined no whole cells were seen, only a few possible fragments

Equal parts of fresh rabbit serum and Tyrode's solution were added to the kidney extracts to make a dilution of 1/50 of virus seeding, two parallel experiments being set up. In the first, the kidney extract treated only with hypertonic saline was used, in the second, the frozen and thawed extract was employed. The virus preparation was a fresh testicular strain of neurovaccine; it was diluted 1/50 in the medium, which was then thoroughly mixed, and distributed in 2 c c. amounts in Carrel and Rivers flasks. Incubation was carried out at 37° C. for 3 to 6 days. In one unfrozen culture, in the course of subcultivation, a total virus multiplication of 10^{20} was obtained, although the dilution of the original virus seeding was 10^{-17}. Virus increase in the frozen medium was most irregular, and it was concluded that a substance released only from fresh cells was essential for the growth of vaccinia virus.

3. Eagles (1935) has repeated some of his previous work. As kidney extract he used the unfrozen extract of Eagles and Kordi (1932). He concluded that there was poor survival of the virus in large numbers of individual flasks, and an inability to secure either growth or survival of the virus in a number of subcultures. Multiplication did, however, take place in two of the series of subcultures

This work has never been confirmed, and it is the opinion of numerous workers that vaccinia will only grow in a medium containing some living cells (e g , Craciun and Oppenheimer, 1926, Maitland, Laing, and Lyth, 1932, Rivers and Ward, 1933 *a*, *b*, Haagen, 1933) It should be noted that Rivers and Ward (1933 *b*), after treating rabbit testis and chick embryo as described by Eagles and Kordi (1932), found some cells not only alive but capable of growth.

The Mechanism of Virus Growth in Tissue Culture

There is no doubt that living cells are present in media of the Maitland and similar [illegible] nd Lyth, 1932). [illegible] d by the followi [illegible] 1929 *a*), heating [illegible] 932–33), treatment with cyanide (Breinl, 1932–33, Maitland and Laing, 1941)

Prior to the introduction of Maitland's medium, it had been shown by Parker and Nye (1925) that growth of vaccinia takes place in intimate connection with the cellular, rather than the fluid, elements of cultures They prepared cultures of virus by the slide technique, rabbit testis being embedded in the center of a large plasma clot. Tests showed the presence of virus in the testis with its corresponding absence from the plasma Later, Muckenfuss and Rivers (1930) suggested that at least one function of the living cells of a tissue culture might be to elaborate a [illegible]

in a preparation in which living cells are present.

Maitland and Laing (1941) have made some interesting observations on the growth of vaccinia in tissue cultures They find that virus increases most rapidly in the first 2–3 days, but growth continues thereafter With larger amounts of tissue, virus multiplies considerably in the first 2–3 days, and continues to increase after 4–5 days Reduction in the amount of tissue tends to lengthen the "lag period." Virus does not grow if the medium is incubated for 5 days before inoculation, yet an inoculated culture incubated for the same time still provides conditions for the continual growth of virus Evidently therefore there is one property needed to initiate growth and another to allow of virus increase The failure of a culture

to initiate growth is presumably due to cell damage. It may be that growth cannot be initiated until virus has combined with a living or surviving cell

Perhaps the factor postulated by Muckenfuss and Rivers serves to keep virus alive, while more intimate cellular contact, as suggested by the work of Parker and Nye, is needed for actual proliferation. The work of Eagles and his associates, who claim to have grown vaccinia in cell-free media, if confirmed, would suggest that a substance similar to that of Muckenfuss and Rivers can be liberated from tissue, and can support actual proliferation of virus even when the cells producing the factor have been destroyed. No such confirmation has yet been reported, however, and it appears that the growth of viruses in tissue culture is intimately dependent on the presence of living cells.

OTHER APPLICATIONS OF TISSUE CULTURE

In addition to their use in the study of virus morphology and for securing virus propagation, tissue cultures can be used for certain other purposes·

1. *Immunization.* Tissue cultures may be used to furnish a growth of virus suitable for immunization of animals, and of man Thus cultures of influenza, common cold, yellow fever, and vaccinia viruses have all been used for human immunization. Rivers and Ward (1935), for example, prepare vaccinia virus suitable for use in human vaccination

(*a*) Routine cultures were ground up in a mortar and inoculations carried out for sterility

(*b*) Bacteria-free suspensions were mixed with equal parts of sterile glycerol, and stored at temperatures below 0° C

(*c*) For use, 0 1 c.c of glycerolated culture virus, diluted 5 to 10 times with sterile saline, was injected intradermally in the skin of the upper arm or thigh, with a 27-gauge needle.

Such cultures have several advantages over the more commonly used vaccines prepared from animal tissues Thus, they can be guaranteed to be sterile, there is a minimum of foreign animal tissue present; and in an emergency a large supply can be rapidly obtained

2 *Isolation of virus* It has been reported that influenza virus may be isolated directly from patients by inoculation of tissue cultures with nasopharyngeal secretion

3 *For* in vitro *neutralization tests.* It has recently been shown that the virus-neutralizing power of immune sera can be titrated by making use of tissue cultures (Magill and Francis, 1938) These workers carried out studies with the virus of influenza, which will now be described into 50 c c Erlenmeyer flasks were pipetted 4 c c of Locke's solution and 5 drops of minced 13-day chick embryos Rabbit antiserum (0 5 c c) was then added. Finally, 0.5 c c of cultured virus was added. Incubation was carried out at 37° C for 48 hours After this time, 0 5 c.c of supernatant fluid was inoculated into a fresh flask containing saline (4 5 c c) and minced embryo (5 drops) only. Now, the final dilution of the original serum was at least 1/100 Incubation was carried out as before, and presence of virus tested by intranasal inoculation of lightly anesthetized mice If virus was destroyed in the first series, then naturally no proliferation occurred in the second, the mice being unaffected.

4. *For studying the interference phenomenon in influenza*

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CHAPTER XIII

THE FERTILE EGG IN THE STUDY OF VIRUSES

THE FERTILE EGG had been studied in connection with various problems (see Goodpasture, 1938), before Rous and Murphy in 1911 made use of the developing chick embryo in their study of the Rous sarcoma. Twenty years elapsed, however, before the method was generally introduced into bacteriology by Woodruff and Goodpasture (1931), in their study of fowl-pox virus

Goodpasture and his collaborators made use of the fertile egg not only in studying virus infections (see below), but also in investigating various problems in bacteriology and serology, in the course of which they showed that the embryo could be inoculated not only on the chorio-allantois but also in the amniotic sac and intravenously. (See Buddingh and Polk, 1937, Gallavan, 1937, Gallavan and Goodpasture, 1937, Polk, Buddingh and Goodpasture, 1938, Buddingh and Polk, 1939, *a*, *b*)

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After about 4–5 days of development, the embryo is seen to be surrounded by the amniotic and chorionic membranes, which develop from extra-embryonic sources The embryo is attached to the yolk sac, containing the yolk, by the volk stalk, at this early stage, the yolk sac fills the greater part of the cavity, along with the smaller albumen sac At this period, the allantois can be detected as an outgrowth from the embryo proper, it is essentially formed of entoderm, with an outer mesodermal layer

As development proceeds, the allantois enlarges, and fuses externally with the chorion to form the chorio-allantois. Internally it fuses with the amnion By about the 10th day, the chorio-allantoic membrane should line the inside of the shell closely At this stage the embryo is of fair size, and lies floating in the amnion, which is surrounded externally by the large allantoic cavity separating the amnion from the chorio-allantois

It may be helpful at this point to describe briefly the various portions of the egg that are of importance in virus work, and at the same time discuss the changes that occur on further incubation

The Shell and Shell Membrane

The shell membrane everywhere lines, and is closely adherent to, the shell. The membrane is tough in consistence and has fibers running roughly in the long axis of the egg, but somewhat obliquely. At the broad end of the egg is the air sac, a well-developed space entirely enclosed by shell membrane, the layers of which divide to form the space. The air sac is only of small size in sterile eggs, but becomes of considerable dimensions in fertile eggs.

The Chorio-allantois

The chorio-allantois is a delicate membrane comprised of chorionic ectoderm externally and allantoic entoderm internally. Between the two layers are numerous blood vessels, capillaries, veins, and arteries, embedded in mesoderm. Two main arteries run from the yolk stalk to the chorio-allantois, the venous return is by two veins associated with the arteries, and by the larger independent allantoic vein.

The chorio-allantois is the main respiratory organ of the embryo, it bleeds readily when damaged. The chorio-allantois is adherent to the shell membrane in all areas, but the two can be separated quite readily.

Levaditi and Grabar (1943) have isolated elementary body-like structures, 200–300 mμ in diameter, from normal chorio-allantoic membranes, and these have to be considered when preparing vaccinial and other virus suspensions.

The Allantoic Cavity

This cavity, which contains 6 c.c. or more of fluid, at its maximum development about the 13th day, is lined by allantoic entoderm, by the time of hatching there is hardly any allantoic fluid left. The fluid is fairly clear, until about the 12th day, when it becomes turbid owing to the presence of urates excreted by the kidneys. Bacterially infected allantoic fluid gives a "false positive" agglutination of chick red cells, which is, however, inhibited by normal serum (Florman, 1946).

Important studies on the antigenic properties of particles prepared from normal allantoic fluid have been made in connection with work on influenza virus.

The pH values of allantoic fluids from noninfected eggs show a rapid drop from the 12th–17th day of development, the Eh of fluid from normal eggs also shows a significant relationship to the state of development, most specimens showing a positive potential (Walker, 1943 *b*, Research Staff, 1945). [For information on the specific conductance and surface tension of egg fluids, see Walker, 1943 *a*, *c*.]

The Amniotic Cavity

The amniotic cavity contains only about 1 c.c. of fluid, which is clear until about the 12th day, when the contents of the albumen sac are discharged into the amnion. The presence of this material causes normal amniotic fluid to agglutinate *certain red cells to a titer of not more than 1/32, chick cells are not agglutinated* (Commission, 1946).

The protein content of the amniotic fluid of uninfected 11-day embryos has been calculated as 0.005 per cent (Nagler, 1946).

The Embryo

A macromolecular lipoprotein complex, containing 10.5 per cent ribonucleic acid has been isolated by means of the ultracentrifuge from normal chick embryo tissue. The sedimentation constant and SG indicate a molecular weight of 4,800,000 approximately, and a diameter of 23 mμ (Taylor *et al.*, 1942). The gain in weight and length of the developing embryo can be expressed in the form of an index (Walker, 1938), Walker's Index $= \frac{1{,}000 \sqrt[3]{\text{wet weight in grams}}}{\text{crown-rump length in cm}}$

CHAPTER XIII

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thin basement membrane can be shown by silver staining. There is a thin submucosal layer of mesenchyme, and then a very narrow layer of smooth muscle fiber.

About the 13th day, clefts appear at regular intervals in the parabronchi, and extend down to the basement membrane; this bulges outward in front of the evaginations from the parabronchial wall. The evaginations grow through and disrupt the muscle cell layer of the parabronchi. The tubular evaginations, or saccules, are lined by a single layer of cuboidal cells. The lumen of the parabronchus communicates directly with the vestibule which leads into the peripheral air capillaries or labyrinth, by the 21st day the labyrinth has completely developed by anastomosis of the air capillaries formed from the earlier evaginations.

The mesenchyme is composed chiefly of undifferentiated stellate cells with a clear cytoplasm and a small, central dark-staining nucleus. The mesenchyme becomes divided into regular hexagonal areas by thin, densely staining endothelial cells linked together to form blood capillaries. Eventually the labyrinth of air

spaces.

Air Sacs

Birds have structures known as air sacs which radiate from the thorax to the abdomen and bones. These organs are not respiratory, but act as "balloons" filled with air. They are derived from the ends of the entobronchi about the 7th day of development, and have the same histological structure, some of the epithelial cells being ciliated

The Albumen Sac

The albumen sac discharges into the amniotic cavity at about the 12th day, through a small communicating duct—the sero-amniotic junction

The Yolk Sac

The yolk, or nutritive fluid, is enclosed in a sheet of vascular, entodermal tissue known as the yolk sac. The interior of the yolk sac is broken up by villous growth, except for a bare area around the yolk stalk The yolk gradually decreases in quantity as development proceeds, and occupies only a small volume at the time of hatching

SOURCE, SUPPLY, AND INCUBATION OF EGGS

Eggs should be obtained from a reliable poultry farm, and delivery should be rate of 80 per cent. should be reached. ion, otherwise they have to be washed

or 3–4 days An egg incubator running at 38°–39° C. is used, but certain workers prefer 37.5°–38° C. for "setting."

Beveridge (1944) has found that embryos incubated at 39° C before inoculation differ from those incubated at 36.6°–37.8° C in the following respects (a) they yield about half the volume of allantoic fluid when infected with influenza; (b) they are not so susceptible to infection with influenza virus injected in the allantoic cavity, (c) chorio-allantoic scarring is more usual He did not find that when influenza virus is injected amniotically or in the yolk sac that the temperature of preliminary incubation has any effect.

The eggs must be turned twice daily, and allowed to cool for 10 minutes on one of these occasions. The tray in the bottom of the egg incubator should be

From the point of view of virus work, interest centers on the histological appearances of the respiratory system up to about the 20th day.

Histology of the respiratory system.

This has been studied by various authors, who give the following account (Gadow, 1893; Juillet, 1911–12; Kerr, 1919; Lillie, 1919; Gallavan and Goodpasture, 1937, Bremer, 1939, Burnet, 1940 *a, b*, Fodden and Rhodes, 1947):

Entodermal diverticula destined to form the lungs grow from the laryngotracheal groove. These diverticula form an elongated air-tube known as the mesobronchus, which extends through all the lung tissue on either side. All the air sacs and passages of the lung develop from the mesobronchus by budding and branching. The buds and branches grow out into primitive mesenchyme.

By the end of the 6th day, several distinct tubular divisions of the mesobronchus are noted, known as secondary bronchi or entobronchi. By the 9th day of development, these in turn divide into innumerable smaller parabronchi which radiate into the rapidly expanding mesenchyme.

By the 11th day, this cellular growth has transformed the lung into an organ composed of epithelial parabronchi embedded in mesenchyme. Developing blood vessels divide the mesenchyme into multiple hexagonal areas, each enclosing a tubular parabronchus.

From the 12th day onward, the parabronchi are the most prominent structures in section They are lined with respiratory epithelium. At about this stage, large numbers of saccular outgrowths develop from the parabronchi.

At this period Burnet (1940 *a, b*) regards the following as constituting a normal appearance (1) a uniform and complete epithelial lining of the parabronchi, (2) the absence of degenerative changes in epithelial cells, apart from some goblet-cell appearances, (3) absence of cells from the parabronchial lumen, (4) the saccules are clearly defined, (5) the mesenchyme is composed of loosely packed uniform cells

The saccules push out into the mesenchyme, and become elongated cul-de-sacs About the 16th day, their ends divide, and continue as narrower tubes which abut onto the surrounding vessels developing in the hexagonal area of mesenchyme. The proximal portion, opening from the lumen of the parabronchus, is known as the vestibule The narrow tubes divide and form a complicated anastomosing labyrinth of fine air capillaries, surrounding the parabronchus, the labyrinth is *developed on about the 20th day There has been considerable* discussion as to whether there is an epithelial lining to the finer air capillaries, and it appears that there is such a layer (Bremer, 1939)

We have with J H. Fodden, studied the histology of the developing respiratory tract, with the object of defining the normal appearances in routine sections, so that pathological changes can be the better understood Our own observations completely confirm and somewhat extend those of Burnet (1940 *a, b*).

During the early days, the mesobronchus is the most prominent structure in sections of lung It extends from the hilum throughout the mesenchyme The mesobronchus is circular, and lined with pseudostratified columnar cells These cells are ciliated, and have a faintly granular cytoplasm with a large pale granular nucleus There is a well-marked basement membrane resting on a narrow reticular submucosa, made up of concentrically arranged spindle-shaped cells, the submucosa contains smooth muscle fibers

About the 7th day, *many entobronchi have been formed by evagination* of the entire wall of the mesobronchus Microscopically the entobronchus resembles the parent mesobronchus, and the epithelial cells are ciliated

The entobronchi, in their turn, give rise to very large numbers of small air tubes or parabronchi These are circular tubes with a broad epithelial lining of tall nonciliated columnar cells, the nuclei of which often show mitoses A complete

thin basement membrane can be shown by silver staining. There is a thin submucosal layer of mesenchyme, and then a very narrow layer of smooth muscle fiber.

About the 13th day, clefts appear at regular intervals in the parabronchi, and extend down to the basement membrane, this bulges outward in front of the evaginations from the parabronchial wall. The evaginations grow through and disrupt the muscle cell layer of the parabronchi The tubular evaginations, or saccules, are lined by a single layer of cuboidal cells The lumen of the parabronchus communicates directly with the vestibule which leads into the peripheral air capillaries or labyrinth, by the 21st day the labyrinth has completely developed by anastomosis of the air capillaries formed from the earlier evaginations

The mesenchyme is composed chiefly of undifferentiated stellate cells with a clear cytoplasm and a small, central dark-staining nucleus. The mesenchyme becomes divided into regular hexagonal areas by thin, densely staining endothelial cells linked together to form blood capillaries. Eventually the labyrinth of air capillaries almost replaces the original mesenchyme of the hexagon From the larger blood vessels fine capillaries penetrate, and are closely applied to, the labyrinth. In the later stages the pulmonary mesenchyme is represented by the sparsely cellular stroma supporting the epithelial structures, pulmonary blood vessels, and lymph spaces.

Air Sacs

Birds have structures known as air sacs which radiate from the thorax to the abdomen and bones. These organs are not respiratory, but act as "balloons" filled with air They are derived from the ends of the entobronchi about the 7th day of development, and have the same histological structure, some of the epithelial cells being ciliated

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The eggs must be turned twice daily, and allowed to cool for 10 minutes on one of these occasions The tray in the bottom of the egg incubator should be

kept filled as directed by the makers In all models of incubator the atmosphere should be kept humid. We have used a gas-heated incubator, and in general have found it most reliable, although an electric one is perhaps preferable.

Hens' eggs are usually used, although ducks' have been employed (Himmelweit, 1938, Beveridge and Burnet, 1946).

"Candling" of Eggs

Eggs are examined by "candling," preferably in a dark room, to see if germination has occurred.

The embryo shows up as a dark area, in contrast to the rest of the egg which is translucent. Movements can usually be detected, and the blood vessels in the chorio-allantois can be readily seen The air sac is clearly visible. Sterile eggs appear translucent. Premature death of the embryo is detected by undue mobility of the shadow and absence of vascular pulsation

Incubation after Inoculation

After inoculation, eggs are incubated in an ordinary bacteriological incubator at 35°–36° C, for 2–4 days, depending on the agent They must not be disturbed during this process. There is evidence that a number of virus agents grows best at this temperature, in preference to 37° C Thus, Sigurdsson (1943) found that embryos incubated at the higher temperature were more resistant to vesicular stomatitis than were those inoculated at 35°–36° C Various similar observations have been made (Beveridge and Burnet, 1946).

INOCULATION OF THE CHORIO-ALLANTOIS

This method was the first to be used in virus work. A number of different methods are available.

Goodpasture's Méthod

This method has been used by Goodpasture and his collaborators (Woodruff and Goodpasture, 1931, Goodpasture, Woodruff, and Buddingh, 1931, 1932, Goodpasture *et al*, 1935, and see Goodpasture, 1938). Twelve-day eggs are candled, and the site of the chorio-allantoic membrane outlined Paraffin is thinly painted on the shell The eggs, resting on plasticine, are then placed in water at 40° C, this reaches up to, but does not touch, the paraffin A window of 1–1½ sq cm is cut in the paraffined surface, using a hard steel trocar (ground with a triangular end and sharp point) The shell is levered off and paraffin painted over the more or less undamaged shell membrane beneath, this is then torn and folded over the cut shell, to expose the chorio-allantois The inoculum is then placed on the chorio-allantois, and a rim of vaseline-paraffin mixture built round the edge of the window, a cover-slip being pressed firmly down on the top Examination of the chorio-allantoic membrane can be carried out through the window from day to day

Burnet's Method

This modification of Goodpasture's method was introduced by Burnet (1933, 1936 *b*), and has been improved from time to time (Beveridge and Burnet, 1946) The air space is first pencilled in, then an equilateral triangle, with 12 mm sides, is marked over the area of densest opacity of the embryo The sides of this triangle are cut through with a rotating disk driven by a dental engine Two intersecting cuts are made over the air sac, which is then pierced with a short, four-sided, sharp steel rod. The triangle of shell already cut is then removed and the shell membrane slit in the direction of its fibers *Slight suction is applied* with a teat to the hole in the air sac In this way the egg contents are displaced and an artificial air space formed between the shell membrane and the chorio-allantois Inoculation of 0 05 c c of material is then made with a sterile Pasteur pipette. The orifice is rimmed and

sealed as in Goodpasture's method, while the air sac hole need not be sealed The egg must be most carefully handled in all subsequent manipulations It is useful to place a small amount of sterile saline on the shell membrane before it is punctured On puncturing, the saline will "seep" in and separate the chorio-allantois from the shell membrane. This process is intensified when suction is applied to the air sac (Burnet and Faris, 1942). Beveridge (1947) describes a technique obviating the use of a drill.

For sealing the egg, cellophane triangles (boiled in distilled water) dipped in sterile egg albumen can be recommended. Adhesive tape is also useful. A somewhat similar method to this has been used by Bengtson and Dyer (1935). These workers cut only two sides of the triangle, bending back the flap to break the third side. Injections are made through the vaseline after the orifice is covered

Stevenson and Butler's (1939) Method

The air chamber and a small circle 1 cm. in diameter on the side of the egg (within the opaque area) are pencilled in. The egg is then placed on plasticine with the inoculation site laterally, the shell over this area is swabbed with rectified spirit and flamed If the air sac is to be pierced, the shell over this area also should be sterilized. Using a sterile scalpel a hole is then drilled through the shell of the inoculation site When the hole is made one should look for the pink chorio-allantoic vessels or slight bleeding, if neither is noted, discard the egg.

The actual inoculation is now carried out by one of the three following methods (*a*) The inoculum is introduced through the hole with a capillary pipette, very little, if any, pressure being applied to the teat. (*b*) First blow in a puff of air, then the inoculum (using separate pipettes). (*c*) After the lateral opening has been made, the air sac is punctured, air is then blown into the lateral hole and finally the inoculum All holes are sealed with paraffin or cellulose acetate in ether or acetone

Another Method

The following method has been recommended (Alexander, 1938, Dunham, 1942, Beveridge and Burnet) An oval opening about 2 × 3 mm. is made by removing the shell with a side-to-side movement of a rotating disc. The area immediately surrounding is brushed with sterile melted paraffin wax The shell membrane is clarified by the smallest possible drop of liquid paraffin. The inoculum is then deposited on the shell membrane, which is punctured through the drop Suction is then applied to the air sac, and the inoculum drawn in The hole is sealed with melted paraffin We have used this method with successful results

Opening and Examination of Eggs

Even with the best technique, some 10 per cent of embryos will die apart from the specific effect of the inoculation After 3 to 4 days, the eggs are ready for examination, the actual time depends on the virus used The eggs should be opened resting on cotton wool soaked in antiseptic, standing in a Petri dish. In Goodpasture's and in Burnet's methods the vaseline and coverslip are removed, and the eggshell broken away with sterile forceps until enough access has been obtained The membrane can be dissected out *in situ* with sterile curved nail scissors In Stevenson and Butler's method the procedure is as follows The egg is rubbed over with rectified spirit from the narrow end to the middle. The narrow end is then dipped into rectified spirit down to one third of the egg The egg is then placed in a metal holder and flamed The sterile (narrow) end projects beyond the holder, and is cracked with a heavy instrument. The shell is picked off the shell membrane with forceps The exposed portion of the shell membrane is then peeled off with another pair of forceps, and the chorio-allantois exposed, with a third pair of

forceps this structure is torn across. The egg is tilted and the contents delivered, the chorio-allantois remains in the shell and is removed into a sterile Petri dish.

Aerobic and anaerobic cultures should be made from every egg opened, if the contents are to be studied further

The membrane removed from the shell is pinned out on a board against a dark background. After fixing in formalin or Zenker's solution, the pocks or other lesions may be counted and examined with a lens or plate-culture microscope. If it is desired to examine paraffin sections of the infected membrane, dehydration and embedding can thereafter be carried out, the membrane being rolled into a compact mass. Sections can be stained by HE and by special methods for inclusions (Buddingh, 1936).

Another histological method is that of Goodpasture, Woodruff, and Buddingh (1931, 1932): Fix in Zenker's solution for 24 hours. Stain the membrane in 2 per cent aqueous acid fuchsin for 10 to 30 minutes; wash. Counterstain with Löffler's methylene blue for 30 seconds. Differentiate in absolute alcohol, clear, and mount in cedar oil. To preserve membranes entire, these may be taken through alcohol, and mounted in plastic solution (Dunham, 1941).

Pathological Changes

Injections of nonirritating fluids should not cause any more than a slight opacity of the chorio-allantoic membrane, but Burnet (1936 *b*) has described the occasional presence of a traumatic lesion, which is more apt to be found in younger than older

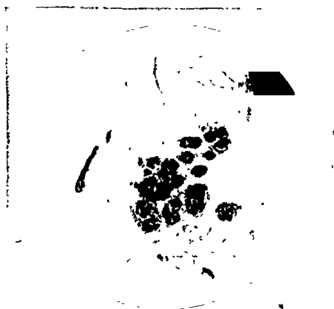


Fig 24 Vaccinial lesions on the chorio-allantois 5 days after inoculation with vaccinia virus, observe the plaques

eggs. It appears as an irregularly thickened opaque area up to 1 cm in length with, microscopically, replacement of ectoderm by granulation tissue, and epithelial downgrowth marginally Nonspecific ulceration is commoner if abrasives are used to prepare inocula.

Goldsworthy and Moppett (1935) found eosinophilic intracytoplasmic and intranuclear bodies in the chorio-allantois unaffected by any virus. D'Aunoy and

Evans (1937) have also drawn attention to the fact that many changes reported in the literature as being specific for viruses can, in fact, be seen in normal eggs Thus they found the following changes to be perfectly normal mesodermal edema and cellular proliferation; endodermal proliferation with vacuolation of cells, which often contained large eosinophilic bodies, probably red cells or degenerated debris; eosinophilic intranuclear bodies were also found

The lesions produced in the egg by genuine viruses, however, are very characteristic, and should not be confused with the appearances just mentioned. Virus dropped on to the chorio-allantois must, unless it be rapidly destroyed, first penetrate the ectodermal layer, here the infection spreads outward with resultant proliferation of infected cells, thereafter, the lesions may assume three main types (Burnet, 1936 *b*)

1. Proliferation of ectodermal cells outstrips the spreading infection, so that a thickened and cornified ectodermal zone surrounds an infected and necrotic center. Burnet states that this type of change occurs with the viruses of ectromelia (incubated at 38° to 39° C.), herpes, influenza, louping-ill, and psittacosis. To the naked eye this change is evident as white areas of dense opacity.

2. In this type the lesion is more destructive, much necrosis occurs, with the [illegible] This type [illegible] is laryngo- [illegible]

3. The ectoderm does not undergo early necrosis, but proliferation spreads out peripherally, and is followed by infection A flat conical lesion of semitransparent appearance, and without central necrosis, results, fowl-pox produces lesions of this type.

In the milder infections the embryo may not be killed, but in the severer types it is destroyed. Virus dropped on the chorio-allantois spreads locally, and may also invade the blood stream, eventually affecting other organs (Anderson, 1940).

Hoffstadt and Tripi (1946) calculated Walker's index (see above) for embryos inoculated with Levaditi's strain of vaccinia. Infected embryos failed to gain as quickly as normals.

Applications of the Chorio-Allantoic Technique

The study of virus morphology.

Numerous workers have found the chorio-allantois a suitable site in which to study the development of inclusion bodies However, in view of the above-mentioned findings of inclusion-like bodies in normal membranes, it would appear as if some, at least, of the bodies described were nonspecific in character. Inclusion bodies characteristic of the following human diseases have been found in stained preparations of [illegible] sittacosis, vaccinia. A [illegible] ectromelia, equine [illegible] laryngotracheitis of [illegible] parrot disease

Himmelweit (1938) has used an ingenious method for studying the formation of virus inclusions *in situ*, working with the duck's egg infected with ectromelia and vaccinia. Briefly, the egg shell was partly cut away and replaced by a coverslip, so that the membrane was visible Microscopic examination was then carried out with a Heine Ultropak (Leitz), which makes use of the principle of annular oblique incidence illumination.

Grafts of human skin, amnion or chorion, already infected with virus, can be placed on the chorio-allantois and the development of inclusions studied by histolog[illegible] 1938) This has been carri[illegible] herpes simplex, variola, and [illegible]

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FIG 24. Vaccinial lesions on the chorio-allantois 5 days after inoculation with vaccinia virus, observe the plaques

eggs. It appears as an irregularly thickened opaque area up to 1 cm. in length with, microscopically, replacement of ectoderm by granulation tissue, and epithelial downgrowth marginally. Nonspecific ulceration is commoner if abrasives are used to prepare inocula.

Goldsworthy and Moppett (1935) found eosinophilic intracytoplasmic and intranuclear bodies in the chorio-allantois unaffected by any virus. D'Aunoy and

The chorio-allantois may be used to test the distribution of virus in experimental animals after various routes of inoculation (Burnet and Lush, 1938).

Titration of antiviral sera.

Eggs may be used in lieu of experimental animals to test the virus-inactivating properties of immune sera. Thus Burnet and Galloway (1934), working with vesicular stomatitis, mixed equal parts of immune serum with falling dilutions of a collodion membrane filtrate of virus. After incubation at 15° to 20° C. for 30 minutes, eggs were injected The usual lethal action of this virus on the embryo was prevented in adequately neutralized specimens. The test proved more sensitive to the presence of unneutralized virus than did a parallel series of injections in the guinea-pig's pad.

When dealing with viruses which produce local pocks on the chorio-allantois, the antiviral properties of immune sera can be tested by their power of diminishing, or actually preventing these lesions. Thus, Keogh (1936) found that antivaccinial sera could be titrated by the percentage reduction effected in the numbers of typical vaccinial pocks, a reduction of 90 per cent. being usually obtained. Burnet (1936 *b*) records a routine method for testing fowl sera for their content of laryngotracheitis antibodies the sera to be tested are mixed with an equal volume of virus (usually diluted 1/10,000), and 1 hour later eggs are injected. Potent sera may completely inhibit the development of the usual lesions.

For the preparation of vaccine for human use.

Egg-cultured virus has been used for human vaccination (see p 382). Goodpasture *et al.*, (1935), for example, giving the following instructions

(*a*) The egg membrane may originally be inoculated with either ordinary lymph, testicular virus, dermal lapine, or directly from the human vaccinial pustule.

(*b*) For the first 3 to 4 passages an incubation period of at least 72 hours is required, although after this period it may be reduced to 48 hours.

(*c*) At least six eggs should be inoculated at each subculture.

(*d*) When the membrane is removed from the egg, a small piece from the center of the lesion should be excised and smears stained for elementary bodies

(*e*) Approximately one-quarter of the lesion is inoculated into dextrose infusion broth and culture carried out. The remainder is placed in the refrigerator to freeze

(*f*) For initiating the next subculture, lesions should be used which show the largest number of Paschen bodies.

(*g*) To maintain the potency of "seed" virus, do not use inocula which have been stored for a considerable period.

(*h*) When titrated, culture virus diluted 1/1,000 should produce a confluent eruption on 90 to 100 per cent of the inoculated area in every test.

(*i*) To prepare vaccine for human inoculation, four parts by volume of 50 per cent. sterile glycerol in saline are added to one part of pulp, pulp is prepared by grinding frozen membrane till finely divided.

Stevenson and Butler (1939) have carried out extensive experiments on the preparation of egg virus

(*a*) Operating inside a special box, vaccinial plaques are removed from the membranes with sterile scissors, after 4 days' incubation.

(*b*) Five to ten plaques may be collected in one dish, weighed, and frozen in the cold room overnight

Grafts of fowl skin have been infected with fowl-pox virus and grafted on the chorio-allantois; after suitable growth, histological material is prepared (Goodpasture and Anderson, 1940). It was found that the epithelium of chickens actively immunized against the virus was resistant while a part of the immunized bird, but became susceptible when grafted on the chorio-allantois.

Cultivation of viruses.

A large number of viruses can be propagated by serial passage through eggs Generally speaking, subculturing is carried out by grinding an infected membrane, and after centrifugation, injecting the supernatant fluid into a fresh egg. Animal or other titrations are usually carried out to prove that there is an actual increase in the virus content of the membrane

Numerous human viruses have been propagated on the chorio-allantois, and the references will be found in the appropriate chapters.

Numerous animal viruses have also been grown.

Titration of viruses.

Virus titrations can be carried out on the chorio-allantois; death of the embryo may be used as the end-point, or the number of pocks may be counted, according to the virulence of the particular strain of virus For any given strain, the relation between the number of virus particles inoculated and the number of pocks produced will be essentially constant; the more virulent the strain in the embryo, the closer to unity is the relation (Beveridge and Burnet, 1946).

Burnet and Ferry (1934), in their studies on fowl-plague and Newcastle disease, inoculated eggs with varying dilutions of virus, and found that infective doses produced death of the embryo within 48 hours. The method whereby the potency of the virus is estimated by counting the number of pocks produced on the chorio-allantois has been carried out for a number of viruses· vaccinia, by Keogh (1936), ectromelia, by Burnet and Lush (1936 *a*); Kikuth's canary virus and fowl-pox, by Burnet and Lush (1936 *b*), and infectious laryngotracheitis by Burnet (1936 *a*) As an illustrative example of the methods used in this class of work, the study of Keogh (1936) on vaccinia may be cited Eggs incubated for 12 days were inoculated with virus, and pock counts made after 48 hours. An initial series of experiments was carried out to determine the approximate strength of virus giving discrete pocks Thereafter, falling dilutions of 3 were prepared, starting at 1/300 and continuing down to 1/24,300, the bulk of inoculum being 0 05 c c. The 1/300 dilution was found to give too many pocks to count accurately, and the pocks in the 1/900 dilution were also somewhat confluent, the 1/24,300 dilution gave an average of 7 5 pocks, the 1/2,700 and 1/8,100 dilutions giving figures of 61 and 21 pocks It was found by a parallel series of intradermal titrations in rabbits that the titers obtained by this method corresponded closely with those by the pock-counting method

Burnet and Faris (1942) found that with vaccinia, the general nature of the relationship was such as to give a series of the type 1–10–62–300 foci for successive tenfold dilutions, with influenza (WS) egg-adapted virus, on the other hand, the relationship was linear

To experienced workers, there is no significant subjective influence in pock counts, with well-defined pocks, the use of 4–6 membranes gives results accurate to ± 50 per cent (Beveridge and Burnet, 1946).

Rabbit myxoma virus can also be titrated on the membrane, and we have worked with fowl-pox with Reed and Crawley in these laboratories

Isolation of viruses.

It has been claimed that influenza virus may be isolated by direct inoculation of eggs with nasopharyngeal secretion (see p 616) Variola can be isolated by these methods (see p 346)

forceps is used to peel off the shell membrane covering the "floor" of the air sac, thus exposing the chorio-allantois, and the underlying allantoic cavity filled with fluid. A small hole is made in the chorio-allantois, avoiding blood vessels The fluid is aspirated with a Pasteur or other type of sterile pipette fitted with a rubber teat. The point of the pipette is passed toward the side of the egg to avoid blockage.

Applications

Allantoic inoculation is widely used in work with influenza virus, Newcastle disease virus will also grow in this cavity, as will certain other viruses

The main applications of the technique are as follows· (1) Passage of laboratory strains (2) Primary isolation from human material (3) To yield a source of crude virus that can be purified for morphological, chemical, and serological studies, and for use as a vaccine. (4) As a source of antigen in serological tests. (5) As a means of titrating virus suspensions or estimating the virus-neutralizing properties of antisera. (6) In chemotherapeutic experiments.

AMNIOTIC INOCULATION

Goodpasture and his collaborators first showed that inoculations could be made into the amniotic sac, by direct vision (Gallavan and Goodpasture, 1937, Buddingh and Polk, 1939 *a*, *b*).

The inoculation of viruses into the amniotic cavity was introduced by Anderson (1940), and Burnet (1940 *a*, *b*, Burnet and Foley, 1941 *a*, Beveridge and Burnet, 1946), and has been widely used, particularly in work with influenza virus. By injecting trypan blue into the amniotic cavity, Burnet (1940 *b*) showed that it was swallowed and passed through the alimentary canal, the mouth and trachea were stained. The object of amniotic inoculation is to introduce material so that it comes in contact with the respiratory tract. Eggs of 13–14 days are usually employed, but for some virus strains younger eggs may be preferred.

Goodpasture's Method

The type of approach is that used for chorio-allantoic inoculation by Goodpasture's method, without any dropping of the membrane. The inoculation is made through the chorio-allantois and amnion into the amniotic cavity with a narrow needle (Gallavan and Goodpasture, 1937, Buddingh and Polk, 1939 *a*, *b*) Anderson (1940) used this method to study the pathological changes produced by herpes

Burnet's Method

Thirteen-14 day eggs are used, and the position of the densest area of the embryo is marked on the shell An oval area 2 × 1 5 cm is drilled over this spot, two further cuts are made running across the oval to make a roughly diamond-shaped area at the air sac end of the oval A hole is then drilled in the air sac, and the diamond-shaped area of shell removed A small slit is made in the shell membrane, a drop of sterile saline may be placed thereon, but is not necessary. Suction is applied with a teat to the hole in the air sac, thus causing the chorio-allantois to drop. The remainder of the drilled oval area is now removed and the exposed shell membrane clipped away The chorio-allantois over the embryo is picked up with a fine forceps (held in the left hand) and a small opening is made with scissors The forceps is now used to pick up the amniotic membrane lying immediately under the opening in the chorio-allantois The amnion is gently pulled through the hole in the chorio-allantois, forming a cone. A Pasteur pipette with a fine point, containing 0 05 c c of inoculum and 0 1 c c. of air is then pushed through the base of the cone, and a little air injected to see if a bubble forms under the amnion If so, the correct cavity has been entered and the inoculation is made The amnion is then allowed to slide back The hole in the shell is sealed

(c) The dish is brought to the laboratory surrounded by freezing mixture. The plaques are ground in a mortar, or a special apparatus, to a pulpy consistence.

(d) To prepare vaccine, add 4 c.c. of 50 per cent. neutralized glycerol in distilled water to each 1 gm. of pulp. Store in sterile ampules, screw-topped bottles, or capillary tubes; perform sterility tests.

(e) Vaccine may safely be stored for 6 to 7 months.

Other applications in the study of virus immunity.

(a) *The theory of the antigen-antibody reaction.* Burnet, Keogh, and Lush (1937) have shown that the egg membrane can be used to furnish evidence on the theoretical problem of the antigen-antibody reaction. They have used pock counting and virus neutralization tests with influenza, louping-ill, vaccinia, and other viruses, and have obtained useful information.

(b) *As a source of antigen.* Infected egg membrane has been used as a source of antigen for the complement fixation reaction in influenza.

(c) *In chemotherapeutic experiments.* The fertile egg can be used for the *in vivo* testing of chemotherapeutic agents in viral (and bacterial) infections (Francis, 1946).

ALLANTOIC INOCULATION

The entodermal lining of the allantoic cavity is very susceptible to certain viruses, influenza in particular, and the method of allantoic inoculation is widely used.

Burnet's Method (1941)

Nine-11 day eggs are candled, the air sac pencilled, and a point marked on the shell where the chorio-allantois is well developed, but relatively avascular. A small groove is then made in the shell with the dental drill, and the inoculation of 0·05–0·2 c.c made with a tuberculin syringe and fine needle. The needle is passed through the shell membrane for a depth of 3 mm., thus penetrating the chorio-allantois and entering the allantoic cavity. The small groove is sealed with paraffin-vaseline mixture.

Eggs are incubated at 35°–36° C. for 2 or more days and then opened as described below. *The eggs should be incubated in egg cups or racks with the air* sac end uppermost

Sometimes leakage may occur unless an additional hole is made in the air sac to allow for expansion. Alternatively, the inoculation can be made through the air sac when the drill hole will be sufficient to allow of expansion.

Another Method

Drill a groove in the shell, as above. Brush an area around the groove about 1 cm square with melted paraffin wax. Expel part of the inoculum of 0·05 c.c. over *the groove Thrust a sterile dissecting needle through the drop, through the shell* and chorio-allantoic membranes, into the allantoic cavity When the needle is withdrawn, the fluid will flow in Then add the remainder of the inoculum which will also be aspirated Sealing is accomplished by a drop of molten paraffin (Burnet and Beveridge, 1946)

Opening of Eggs

At the completion of incubation, the eggs are placed in the ice chest for 4–18 hours, to kill the embryo and prevent bleeding The shell in the region of the air sac is sponged with methylated spirit, and the shell drilled through. The top of the egg is then removed with a sharp scalpel or razor blade. A pair of small sterile

Changes Produced in the Embryo

Severe changes are produced in the respiratory tract by influenza virus (see Ch. LIX) and the virus of Newcastle disease of fowls (see Ch LIII)

Herpes virus produces nuclear inclusions in the tissues of the amnion and the skin of the embryo Proliferation and subsequent vesiculation of the cutaneous and pharyngeal epithelium may be noted Invasion of the blood stream may occur (Anderson, 1940). Mumps virus also grows [illegible] multiply and pro- d[illegible]. Laryngotracheitis [illegible] which present the features of epithelial proliferation, formation of intranuclear inclusions, and degeneration without gross necrosis. Psittacosis multiplies freely but produces relatively slight changes, almost confined to the superficial epithelial cells of the larger bronchi

Applications

Amniotic inoculation is extensively used in the study of influenza virus Its main applications are as follows (1) Passage of laboratory strains (2) Primary isolation from washings (3) Titration of virus suspensions

YOLK SAC INOCULATION

Cox's Method

The method of yolk sac inoculation has been chiefly popularized by the work of Cox (1938 *a*, *b*, *c*, 1941).

Eggs are incubated for 5–6 days. They are inoculated in the yolk by passing a narrow gauge needle 1–1½" long into the center of the egg through a groove drilled in the shell of the air sac The inoculum can be up to 0 5 c c. or even 1 0 c c in bulk

Incubation is then carried out at 35°–36° C. Virulent agents invade the embryo and may cause death The yolk sac is removed by drilling away the air sac, and removing the shell membrane and chorio-allantois in its floor. The contents are then gently decanted into a sterile Petri dish If desired, the embryo can first be pulled out and the yolk sac then delivered by traction on the umbilical stalk (Beveridge and Burnet, 1946).

Passage is usually carried out with a 10 per cent. suspension of ground yolk sac tissue, or with yolk itself.

Application

A number of viruses grows readily in the yolk sac, and form characteristic elementary bodies that can be readily detected in smears made of the lining membrane e g , psittacosis, LGI, and pneumonitis viruses.

Typhus rickettsiae grow well in the yolk sac

Yolk sac material is used as a source of vaccine, and as antigen for serological tests in psittacosis, LGI, pneumonitis, and rickettsial infections

Yolk sac inoculation is also of value in the primary isolation of agents of the psittacosis-LGI group

The method can be used to study the effect of drugs and antibiotics on viruses, such as those of the LGI-pneumonitis group An effect of the drug is shown by a delay in the death of the embryo (Hamre and Rake, 1947)

INTRAVENOUS INOCULATION

Embryos can be inoculated intravenously (Polk, Buddingh, and Goodpasture, 1938, Buddingh and Polk, 1939 *a*, Burnet and Beveridge, 1946) The inoculation

with a coverslip resting on a wall of paraffin-vaseline Incubation is carried out at 35° C. for 2–4 days.

In opening the egg after incubation, the shell is chipped away, and the shell membrane cut away, to the margin of [illegible] fluid may be removed if required, and [illegible] egg. Some amniotic fluid is removed w[illegible]

In influenza work, the embryo is then removed to a sterile Petri dish, and the contents of the trachea aspirated with a suitably drawn-out and slightly bent Pasteur pipette In infected embryos the fluid is turbid, normally it is clear. The fluid is centrifuged in a capillary, and a Leishman-stained film made from the deposit.

The trachea and lungs can then be dissected out for histological examination We ourselves prefer to fix the embryo entire for such an examination.

If passage or titration experiments are to be carried out, the lungs are removed with sterile precautions and ground with powdered abrasive in 1 c.c. of broth. Passage can also be performed with ground tracheas.

Burnet and Beveridge (1946) have described an improved method of locating the embryo. The egg is candled and the site of the main allantoic vein pencilled. This vein can be seen near the air sac, or its position inferred from the convergence of two large veins. The egg is held with the air sac end towards the operator, and a line is traced back from the allantoic vein to the broadest diameter of the egg. The inoculation site lies on this diameter, but ½″ away from the line in a clockwise direction.

We use a simplified method based on that of Goodpasture and Burnet (Rhodes, 1946). The egg is candled, and an equilateral triangle marked overlying the embryo. The triangle is drilled and then removed A small drop of sterile saline is placed on the exposed shell membrane, which is then punctured. The saline helps to separate the chorio-allantois. The shell membrane is clipped away to expose the chorio-allantois. This is incised by a touch of a hot needle, which acts as a hemostatic. The amnion is pulled through this hole with a delicate pair of forceps held in the left hand. The inoculation is then made with a syringe and fine needle held in the right hand This method obviates the necessity for "dropping" the chorio-allantois

Hirst's (1942) Method

In this method, the inoculation is performed over an egg candler. The egg is rotated till the shadow appears near the upper surface One puncture is then made in the shell of the air sac and another overlying the embryo. The inoculation is made through the latter hole with a 22 gauge, 1″ long needle, thrust quickly in, nearly to full length. If the embryo is hit, the shadow disappears, but can be made to reappear by moving the needle The inoculum is 0 2 c c.

By this method, some of the inoculum, if not all, presumably goes into the tissues of the embryo, rather than directly into the amniotic cavity.

Taylor and Chialvo's (1942) Method

Remove the shell and shell membrane overlying the air sac and expose the floor Render the floor translucent with a few drops of spirit, so that the border of the underlying yolk sac and blood vessels can be noted. Avoiding these structures, plunge the points of a pair of fine forceps through the two membranes and fairly deep down Open the points to grasp the amnion and withdraw a small "tent" through the hole Inoculate into the base of the exposed amniotic fold and let it fall back into place Cover the air sac with a suitably shaped glass cup Incubate in an upright position. To examine the embryo, remove the cup, cut away the shell membrane and chorio-allantois, lift out the embryo with forceps, and place in a Petri dish Allantoic fluid may first be aspirated

and this prop-
to egg yolk
10-day em-
bryos is less anaphylactogenic than that from other embryos (Berge and Hargett). Formalin reduces somewhat the danger of allergic reactions (Randall, Mills, and Engel, 1947).

4. Sensitivity to chick and egg antigens in human beings may follow inoculation with whole embryo vaccines (Stull), these vaccines contain egg yolk, yolk sac, and chick embryo antigens, but very little egg white.

5 Using the Prausnitz-Kustner passive transfer technique, Stull found that typhus vaccine gave positive skin reactions in high dilution

6. Highly purified rickettsial bodies do not appear to contain significant amounts of egg antigens.

7. Ideally, a skin test with egg and chick antigens should be performed before *inoculating persons with egg vaccines. The vaccines known to contain egg and* chick antigens should be avoided in persons giving a history of susceptibility to egg or chick. In an important study, Rattner and Untracht (1946) review a number of instances where serious and even fatal allergic manifestations have followed the administration of egg vaccines to sensitive persons. In a group of allergic children, they found that the intradermal inoculation of 0·02 c c of allantoic fluid vaccine served to identify the dangerously sensitive persons In those giving a severe reaction to the skin test, adrenalin should be given along with the dose of vaccine; where the skin reaction is very extensive, the vaccine should not be given at all.

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can often be made in a large vein crossing the floor of the air sac, after removal of the shell, if the shell membrane is clarified with a drop of sterile liquid paraffin Alternatively, a large vein can be located in the superficial chorio-allantois by candling. The direction of flow of blood is ascertained if possible. The shell overlying this area is removed, and a drop of liquid paraffin applied to clarify. A 27-gauge needle is then inserted acutely, in the direction of flow; the needle should rest on the shell.

To obtain blood after intravenous inoculation, the needle can be inserted in the opposite direction. Alternatively, a large vessel under the floor of the air sac can be nicked, when the blood will collect on the shell membrane. If desired, the embryo can be dissected and heart blood aspirated.

INOCULATION BY OTHER ROUTES

1. *Intracerebral inoculation* can be performed (Buddingh and Polk, 1939 *a*, Anderson, 1940, Dawson, 1941). The essential of this method is accurate location of the head by candling. A window or triangle is cut in the usual way, and 0 05 c.c. inoculated into the brain with a 27-gauge needle inserted with a sharp jab. Anderson (1940) fou
tion of herpes virus,
even from there alo
Dawson found Negri bodies.

2. *Intra-ocular inoculation* can also be carried out (Dawson, 1941).

3. *The albumen sac* can be inoculated (Polk, Buddingh, and Goodpasture, 1938).

4 *The tissues of the embryo* can be inoculated, either by the usual type of ap-
...ed into the embryonic tissues
...needle into the center of the
1937, Polk, Buddingh, and Goodpasture, 1938, Buddingh and Polk, 1939 *a*) An intraperitoneal injection can be given by pulling out a leg and passing the needle through the thigh (Polk, Buddingh, and Goodpasture, 1938). Anderson (1940) infected embryos with herpes by scarification, and by subcutaneous inoculation, producing characteristic histological changes locally. Peritonitis, and ascending myelitis might occur as the result of the spread of the virus.

Vaccines can be prepared from minced whole chick embryo tissue and are used in yellow fever, and equine encephalomyelitis.

In large scale vaccine production it is useful to use mechanical aids for opening and removal of embryos such as described by Pickels (1942), so that a thousand eggs or more can be handled in an hour.

EGG VACCINES

Vaccines prepared from the egg are being increasingly used, the best known are influenza vaccine from allantoic fluid, typhus and spotted fever vaccines from yolk sac, and encephalomyelitis and yellow fever vaccines from whole embryo

Egg vaccines possess many advantages, and in particular the risk of contamination with bacteria or latent viruses in animal tissues is greatly minimized It is important to realize, however, that these ... sensitize guinea-pigs and human beings to ...ckoff, and Bohnel, 1941, Berge and Hargett, 1... 1946, Stull, 1946) The more important observations are as follows

1. It is rare for normal uninoculated adults to give a skin reaction to typhus (yolk sac) vaccine, egg white, or egg yolk (Plotz)

2 A course of yolk sac vaccine can induce skin sensitivity to egg antigens (Plotz).

SECTION 2. DISEASES OF THE SKIN AND MUCOUS MEMBRANES

CHAPTER XIV

MOLLUSCUM CONTAGIOSUM

BATEMAN (1817) of London has been credited with being the first physician to recognize the disease clinically. Paterson (1841), on the other hand, suggested that the disease was probably noticed earlier, about 1793, by Tilesius Bateman (1817) made a reference to the latter, but called Tilesius's case one of molluscum pendulum Bateman's illustration showing a case of molluscum contagiosum affecting the face of a young woman is typical of the condition, but it appears to be different from his picture showing molluscum pendulum, and so in all probability the two diseases may have been dissimilar.

CLINICAL AND PATHOLOGICAL FEATURES

Molluscum contagiosum usually commences as a pimple in the skin which becomes red, tender, and swollen, and gradually develops into a small yellowish-pink tumor varying in size from a pin-head to a hazel nut. The skin over its surface is tightly stretched and shiny, and usually presents a central dimple. Such lesions are liable to appear on any part of the skin—the face, arms, buttocks, back, and sides being probably the commonest sites for their occurrence, less commonly the genitalia, margins of the eyelids, or scalp become involved The tongue and mucous membrane of the mouth are seldom affected, and the soles of the feet and hands are said never to be attacked The molluscum tumor frequently undergoes suppuration, and scratching of the area may spread the disease to healthy parts of the body.

Molluscum of the eyelids may be complicated by a secondary conjunctivitis and keratitis, microscopical sections show the presence of typical inclusion bodies (von Papolczy, 1933, Quill, 1940, Magnus, 1944).

Inoculation of filtered or unfiltered molluscum tissue extracts into the skin of human volunteers produces lesions after a long incubation, stated by Juliusberg (1905) to be 50 days, by Wile and Kingery (1919) to be 14 to 25 days, and by Findlay (1930) to be 35 days

Molluscum is more common in children than adults. A section made through the center of a nodule shows a down-growth of superficial epithelium extending into the deeper layers of the skin The cells are grouped into little bundles, and the center of the collection is occupied by a mass of hyaline degenerated epithelial cells Each of these cells is enlarged to three or four times its normal size, the nucleus being pushed to one side, and the cytoplasm occupied by a comparatively large hyaline oval mass, the Henderson-Paterson (1841) or molluscum inclusion body

EPIDEMIOLOGY

Geographical distribution The condition has been recognized by clinicians in different parts of the globe, and since its original description in England cases have been reported from countries as widely separated as Germany, India, France, Holland, Spain, the United States of America, French West Africa, the Belgian Congo,

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SECTION 2. DISEASES OF THE SKIN AND MUCOUS MEMBRANES

CHAPTER XIV

MOLLUSCUM CONTAGIOSUM

BATEMAN (1817) of London has been credited with being the first physician to recognize the disease clinically Paterson (1841), on the other hand, suggested that the disease was probably noticed earlier, about 1793, by Tilesius Bateman (1817) made a reference to the latter, but called Tilesius's case one of molluscum pendulum Bateman's illustration showing a case of molluscum contagiosum affecting the face of a young woman is typical of the condition, but it appears to be different from his picture showing molluscum pendulum, and so in all probability the two diseases may have been dissimilar.

CLINICAL AND PATHOLOGICAL FEATURES

Molluscum contagiosum usually commences as a pimple in the skin which becomes red, tender, and swollen, and gradually develops into a small yellowish-pink tumor varying in size from a pin-head to a hazel nut The skin over its surface is tightly stretched and shiny, and usually presents a central dimple. Such lesions are liable to appear on any part of the skin—the face, arms, buttocks, back, and sides being probably the commonest sites for their occurrence; less commonly the genitalia, margins of the eyelids, or scalp become involved The tongue and mucous membrane of the mouth are seldom affected, and the soles of the feet and hands are said never to be attacked The molluscum tumor frequently undergoes suppuration, and scratching of the area may spread the disease to healthy parts of the body.

Molluscum of the eyelids may be complicated by a secondary conjunctivitis and keratitis, microscopical sections show the presence of typical inclusion bodies (von Papolczy, 1933, Quill, 1940, Magnus, 1944).

Inoculation of filtered or unfiltered molluscum tissue extracts into the skin of human volunteers produces lesions after a long incubation, stated by Juliusberg (1905) to be 50 days, by Wile and Kingery (1919) to be 14 to 25 days, and by Findlay (1930) to be 35 days

Molluscum is more common in children than adults. A section made through the center of a nodule shows a down-growth of superficial epithelium extending into the deeper layers of the skin The cells are grouped into little bundles, and the center of the collection is occupied by a mass of hyaline degenerated epithelial cells Each of these cells is enlarged to three or four times its normal size, the nucleus being pushed to one side, and the cytoplasm occupied by a comparatively large hyaline oval mass, the Henderson-Paterson (1841) or molluscum inclusion body.

EPIDEMIOLOGY

Geographical distribution The condition has been recognized by clinicians in different parts of the globe [illegible]
been reported from co
land, Spain, the Unitec

the Philippines, and Italy. Therefore, under present-day conditions of travel, the disease can be assumed to possess a world-wide distribution (see von Prowazek, *1911*, *Spillman et al.*, *1930*, *Chiyuto*, *1932*; *Ghosh*, *1934*, *Rossi*, *1935*; van den Berghe, 1937, Low, 1946).

Mode of transmission. Infection is spread from case to case by direct contact, and numerous reports to this effect are on record. Paterson (1841) quotes a case in which lesions developed on the breast of a nursing mother, acquired from similar nodules on her infant's face. He also mentions another instance, in which a man with lesions on his penis communicated the disease to his wife, who developed similar tumors on the vulva; *Nardie (1934) describes an outbreak among a group* of wrestlers Sutton and Sutton (1935) depict an illustration of a patient who contracted multiple lesions on the skin, presumably after being tattooed with an infected needle, Paton (1909) and Spillman *et al.* (1930) reported cases acquired following accidental surgical infection. A case of molluscum contagiosum acquired through coming in contact with an infected woolen garment has also been reported in the literature. The condition can be spread by the hands of cosmetic salon attendants or by masseurs.

Molluscum contagiosum is a relatively common skin disease in Edinburgh, and the figures of Graham Little (see Walker and Percival, 1932) showed that a high proportion of cases occur among those frequenting public swimming baths. The distribution of lesions on the skin of such patients suggested that the infection is acquired through contact with the gymnastic exercising equipment in the swimming baths. The suggestion has also been made that the condition may be carried by some unknown animal or insect vector, and Ehrmann (1892), for example, has incriminated the crab louse *Pediculus pubis*, but this report lacks confirmation Interest in the possible role of animal carriers was aroused by the work of Peterkin (1937), who drew attention to a disease closely resembling molluscum, contracted by shepherds who had handled flocks infected with "Orf," or contagious pustular dermatitis (Rabagliati, 1927, Aynaud, 1923, Howarth, 1929, Berry, 1901, Glover, 1929–30, Selbie, 1944, 1945, 1946 *a*, *b*, Horgan and Haseeb, 1947). Schoch (1939) in America has described a case similar to those of Peterkin, but likens it to sheeppox or cowpox In view of the work of Selbie, Horgan and Haseeb, and others it appears unlikely that the

In view of Peterkin's
material, by corneal scarifi
under observation for six months, but at the end of this time the result was negative

PROPERTIES OF THE VIRUS

Lipschutz (*1911*) measured the elementary bodies (which he called *Strongyloplasma hominis*) occurring in this disease and found that they measured 0 25 μ in diameter in Giemsa-stained films One of us stained the elementary bodies by Paschen's method, and estimated their size to be 0.3 μ when measured by direct photomicrography, and 0 35 μ when calculated by micrometric extinction (van Rooyen, 1938) Electron micrographs show the EB's to be brick-shaped (Ruska and Kausche, 1943). Boswell (1947) found the size to be 302×220 mμ

Recently, we attempted to agglutinate these elementary bodies with the sera of human patients, although a number of tests was performed, we failed to demonstrate the presence of specific agglutinins in these sera The absence of these antibodies may be attributable to the fact that, since the virus grows in the superficial skin epithelium, and does not gain access to the general circulation, it consequently fails to provoke an antibody response This opinion is shared by certain dermatologists, who have asserted that there is no evidence of immunity to molluscum contagiosum, and unless the condition is treated by surgical excision of the lesions, there is no reason why it should not continue indefinitely (Walker and Percival, 1932).

The virus was shown to pass the Chamberland filter by Juliusberg (1905), a Berkefeld filter by Wile and Kingery (1919), and the Berkefeld V and Pasteur-Chamberland and L_1 filters by Findlay (1930). The latter found that the virus retained its activity after preservation in 50 per cent glycerol for one month. Walker and Percival (1932) state that, if a molluscum nodule on human skin is pricked with a needle dipped in carbolic acid, or exposed to the action of x-rays, the condition rapidly heals. It is difficult, however, to decide from such scanty data whether these agents bring about recovery by killing the virus or by stimulating local tissue reaction.

ANIMAL INOCULATION EXPERIMENTS

Among other workers, Goodpasture and Woodruff (1931) attempted to cause infection of rhesus monkeys, as well as other laboratory animals, but with negative results. We ourselves tried to infect a rhesus monkey by intracorneal and intracerebral inoculation, but without success, ferrets and a sheep likewise proved negative. Findlay (1930) reported similar negative results after inoculation of monkeys, anthropoid apes, fowls, and pigeons. Ota and Huang (1934) claimed to have been able to infect rabbits by intratesticular inoculation, but their work lacks confirmation.

Maplestone and Panja (1939) claim to have transmitted the infection to rabbits by the cisternal inoculation of a filtrate prepared from a nodule. The animals died with nervous symptoms.

THE MOLLUSCUM INCLUSION BODY

This is an acidophilic intracytoplasmic structure that is usually circular or pear-shaped, measuring from 20 to 37 μ in diameter, and surrounded by a definite membrane. A number of contradictory views has been expressed with regard to its probable significance, and it was originally thought by Lipschutz (1911) and von Prowazek (1911) to be a parasitic protozoon. On the other hand, Kromayer (1893)

desiccation and fusion of the vacuoles within the cytoplasm of the infected epithelial cell. At the present time, the condition of molluscum contagiosum is regarded as a virus infection, and the molluscum body as a typical inclusion body. Many observers have shown that the molluscum body contains innumerable minute granules, these were originally called *Strongyloplasma hominis* by Lipschütz, their discoverer, but are now recognized to be typical elementary bodies, similar to those found in other virus diseases. Later, Goodpasture and Woodruff (1931) performed comparative micromanipulation studies on the inclusion bodies of fowl-pox and molluscum, they found that, after the cell epithelium had been digested with trypsin, the molluscum inclusions had a sticky covering which resisted dissection with a Chambers' micromanipulator.

According to one of us (van Rooyen, 1938), the molluscum body grows from a minute elementary body which enters the cell. He does not agree that the inclusion is developed from fusion and desiccation of the vacuoles in the cytoplasm of the infected epithelial cell, and suggests that the vacuoles are formed by degenerative changes set up within the cell, due to the growth of the inclusion body therein. A number of observations was also made (van Rooyen) on the shape, size, and structure of the inclusions, it was shown that they may be either small and circular in outline, measuring about 20 μ in diameter, or alternatively, larger oval or pear-shaped in contour, attaining a maximum size of 37 μ in their long axis, it was suggested that these larger forms probably represent the more mature developmental stages of the parasite. The large pear-shaped molluscum bodies were

also demonstrated to possess a surrounding membrane, thickest at the broad segment end, and thinnest at the other pole, where it resembled a cap

By microdissection it was proved (van Rooyen) that the conical thin-walled pole of the body could be readily ruptured by incision with a microdissecting needle point, and that the interior of the molluscum body contained numerous elementary bodies suspended in a gelatinous fluid.

We believe that, in miniature, the molluscum body presents certain morphological resemblances to the sporangium of a fungus parasite such as *Rhinosporidium seeberi* (Wernicke, 1903, Ashworth, 1923); this parasite causes a polypoidal affection of the nasal mucosa, and is accompanied by the formation of large cysts in the tissues Like the molluscum body, the *Rhinosporidium* cyst has also a wall or membrane which possesses a weakened exit pore, and contains numerous spores suspended in a mucoid substance In view of these new findings, the question arises *as to what is the precise biological classification of the molluscum inclusion body*, but much further work is necessary before the matter can be discussed fully.

The chemical composition of the molluscum body has recently been investigated by one of us and the results have suggested that the outer covering of the inclusion is probably composed of carbohydrate material Thus, after staining the inclusion bodies with iodine solution the color could be made to disappear by warming the slide, and reappear by permitting to cool Furthermore, the iodine-reacting properties of the molluscum body could be permanently removed by subjecting films to the digestive action of fresh human saliva, but not boiled saliva Microincineration studies were also conducted, and it was found that the concentration of calcium salt in the ashed inclusions was greater in amount than in the surrounding body tissues (van Rooyen, 1939).

LABORATORY DIAGNOSIS AND SPECIFIC TREATMENT

If the area of the skin bearing the tumor is tender, it should be anesthetized with cocaine or frozen with carbon dioxide snow, before attempting to avulse the lesion with forceps The nodule should be squeezed with the forceps, the milky exudate placed on a glass slide, a drop of liquor potassii added, a coverglass placed over it, and the preparation viewed under the dry high-power objective of the microscope If molluscum bodies are present, they are usually found in large numbers If it is desired to stain the bodies in wet films, to render them more conspicuous, we recommend that a drop of Lugol's iodine should be substituted for liquor potassii solution, after such treatment the bodies will appear as large, oval, deeply stained brown masses A drop of 1 2,000–25,000 dilution of brilliant cresyl blue made up in 0 86 per cent saline solution gives even better results, the dye exhibits a selective staining action on the molluscum body, which appears as a large blue mass inside the cytoplasm of the infected epithelial cell

Maplestone and Panja (1939) claim to have demonstrated fixation between convalescent serum and antigen prepared from infected rabbit brain

There is no specific treatment Brain (1937) has tried vaccines consisting of an extract made from molluscum lesions, but has failed to show any therapeutic benefit. It has been found that the oral administration of sulfonamide has an inhibiting effect on the progress of the lesions (Somerville, 1941, Hill and Downing, 1942, Laymon, 1946).

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CHAPTER XV

WARTS (VERRUCAE)

CLINICAL FEATURES

THE INCUBATION PERIOD is long, as has been proved by various observers who have carried out human inoculation experiments with wart material and found the following periods to elapse before the development of growths 1 month (Wile and Kingery, 1919), 6 months (Kingery, 1921); 8 months (Findlay, 1930); 90 days (Brunschwig, Tschetter, and Hamann, 1940); and 20 months (Templeton, 1935).

A number of types of wart varying somewhat in appearance, selective site, and age preference is described. It is becoming increasingly evident, however, that the etiological agent is the same in all, and that inoculation of material from one type of wart can, under suitable conditions, give rise to those of another type

(*a*) *Common warts* occur mainly on the hands as discrete, rounded, or oval growths of gray color and dry appearance. On occasions they are found elsewhere on the skin A modification of this type of wart is found on the palms or soles (palmar or plantar warts) The plantar wart occurs in athletes (Sutton and Sutton, 1935), and in schoolchildren, particularly girls (Halberg, 1936, Lomholt, 1936, MacKenna, 1938). The weight of the body causes this type of wart to become depressed below the surrounding skin level, and so to occupy a cup-shaped cavity covered with a hard epidermal "lid." This lid can be removed to disclose a soft necrotic center which overlies the skin papillae. Plantar warts are favored by flat feet, and the lesions occur chiefly on the ball of the foot and the heel

A special form of the plantar wart is the mosaic wart In this condition there are small, dry warts which are very resistant to therapy.

(*b*) *Juvenile* (*plane*) *warts* are smaller than the above and occur together in groups. In shape they are flatter than the common wart and are most commonly found on the face and dorsum of the hand

(*c*) *Digitate warts* are pedunculated growths broken up into leaf-like folds, each tipped with hardened epithelium, they are found on the face and scalp

(*d*) *Filiform warts* are small delicate tufts of growth found on the eyelids and sometimes the neck They are common on the neck in women.

(*e*) *Genital warts* It has been suggested (Wilson, 1937) that this term should be used to cover those conditions often known variously as moist warts, fig warts, gonorrheal warts, venereal warts or vegetations, and condylomata lata At one time it was thought that such warts were of different etiology to ordinary skin warts, but it has been shown repeatedly, following the original work of Waelsch in 1918, that this is not the case This observer inoculated genital wart material intradermally, with production of a typical plane wart Despite this observation many venereologists and others still contend that gonorrhea may cause some of these warts, but it is more likely, however, that the only effect of gonorrhea is that the purulent discharge causes irritation of the wart with consequent increase in size, and perhaps superficial ulceration Genital warts increase in size during pregnancy but decrease thereafter. In the male they appear as small grayish rough nodules in the coronal sulcus or subpreputial region In the female they are found on the labia and around the vulval orifices and, owing to the greater moisture of the parts, assume large [illegible] ion occurring [illegible] in the anal and axillary regions,

(*f*) *Laryngeal papillomata* may be single or multiple, either flat-based or pedunculated, and white or red in color, the surface is rough and mamillated The classi-

cal work on the etiology of this condition is that of Ullmann (1923) who demonstrated the following points he reproduced a flat wart on his own arm with an extract made from a laryngeal papilloma; he reproduced flat warts on the skin of the face in 3 out of 4 children, he demonstrated the filtrability of the virus (although he did not specify the filter used), he transmitted the infection to the vagina of a bitch, and in sections of this lesion described intranuclear eosinophilic inclusions similar to those of herpes febrilis. Ishikawa (1936) has reproduced warts in man and in the oral and vaginal mucosae of dogs with extracts from human laryngeal papillomata, thus confirming the original work of Ullmann

HISTOLOGY

Full histological descriptions of warts are to be found in the works of Unna (1896) and McCarthy (1931).

The Common Wart

Unna recognized 3 distinct stages of every wart

(*a*) *Stage of commencement.* Simultaneous acanthosis and hyperkeratosis result in the affected papillae being depressed, flattened, and stretched. The earliest stage is, therefore, that of a pure acanthoma

(*b*) *Stage of acme.* Hitherto the cutis has remained comparatively unaltered, but now epithelial ridges begin to grow inward. The cutis responds with inflammatory changes of a mild character, and opposes the entrance of the ridges, which become bent This growth of the epithelium results in a change of shape of the papillae, which acquire a broad fixed end and combine into a common papillary trunk. The prickle cells are active, considerably swollen, and show numerous mitotic figures, the granular layer also is thicker than normal. Between the papillae there is an increased granularity due to an exaggeration of the normal process of formation of keratohyalin.

(*c*) *Stage of regression.* There is no further growth of prickle cells, but cornification continues and extends deeper and deeper, tears and cracks appear, and some horny tissue is cast off. Eventually the papillae are disclosed, covered in horny tissue and resembling expanded fingers which turn out, thus drawing attention to their constricted bases.

Juvenile Warts

The histological changes are less severe than the above and are limited entirely to the epithelium A considerable hyperkeratosis is found at all stages Acanthosis is of slight degree at first but becomes more and more pronounced with age (McCarthy, 1931)

Genital, Filiform, and Digitate Warts

These warts have a somewhat similar histological appearance (Unna, 1896)

(*a*) There is a patchy thickening of the epithelium which projects in one or more points, due to swelling of the subjacent cutis

(*b*) In both common and genital warts the preliminary acanthosis flattens out the papillae In the former this condition persists, but in the latter the number of papillae steadily increases by branching, a chronic inflammatory cellular exudate is found in these new-formed papillae.

(*c*) The covering epithelium shows very active growth of the prickle cells, but there is no hyperkeratosis, consequently, marked furrowing of the surface results, with shedding of the horny layer, and this accounts for the soft appearance of larger genital warts

SPREAD OF WARTS

It has long been known by the laity that if warts are made to bleed, then spread is liable to occur to neighboring parts of the skin The infectivity of warts was

originally proved experimentally by workers many years ago (e.g., Variot, 1894; Jadassohn, 1896, Juliusberg, 1903). Warts may be spread from case to case by direct contact; in shaving, by water; by flooring, e.g., in bathing-pools and school lavatories (Lomholt, 1936), by the chiropodist's instruments if improperly sterilized (Sutton and Sutton, 1935); and by the hairdresser's brushes and combs (Cranston Low, 1934). A group of related cases occurred in a factory where glue had become infected with the virus from one of the workers (McLaughlin and Edington, 1937). Genital warts are spread venereally.

ANIMAL WARTS

Warts of similar appearance to those of human beings occur naturally in dogs and cattle. The virus of dog warts is known to be filtrable, but is not transmissible to man (Findlay, 1930) The virus of cattle warts is also filtrable (Magalhães, 1920), as regards transmission to man, Schultz (1908) obtained positive results, but Soule and McKinley (1930–1) failed to confirm his observations.

EVIDENCE FOR A COMMON ETIOLOGY OF WARTS

Human warts. On clinical grounds there is considerable likelihood of the similarity, if not actual identity, of the infecting agents of the different types of human wart, for the following reasons common and plane warts are frequently found together in the same patient; genital warts and other types of wart frequently coexist, laryngeal papillomata are frequently associated with plane warts of the face. Scientific investigations have shown that genital warts (Waelsch, 1918) and laryngeal papilloma extracts (Ullmann, 1923, Ishikawa, 1936) both give rise to flat warts on intradermal injection. As regards plantar warts, the general consensus of opinion is that these are due to the same agent as the other types, being frequently coexistent. It has, however, been suggested by Goldsmith (1936), who saw an epidemic of plantar warts without any other lesions, that the virus may be distinct. *It should also be noted that warts of any variety frequently* "breed" true to type in any one person.

Animal warts. These warts do not appear to be transmissible to man and probably there is a specific virus, both for dog and for cattle warts

PROPERTIES OF THE WART VIRUS

Animal inoculation. Despite numerous attempts to transmit the virus to animals, the only definite results have been obtained in the case of laryngeal papillomata, Ullmann (1923) and Ishikawa (1936) have transmitted this infection to the vagina of bitches Wilson (1937), working with genital warts, has, however, suggested that the rabbit's testicle may prove a favorable site for injection, and this should be further investigated It has not proved possible to infect the rabbit by cutaneous, subcutaneous, or mucosal inoculation (Brunschwig, Tschetter, and Hamann, 1940, Callomon, 1942) The virus of verrucae may on occasions hasten the malignant degeneration of "warts" produced on the rabbit's ears by benzpyrene (*Brunschwig, Tschetter, and Hamann, 1940*)

Morphology and filtrability Elementary bodies have not been described With regard to inclusion bodies, the increased eosinophil granularity of the superficial layers of the wart has already been considered These granules have undoubtedly deceived certain workers who have suggested that they are specific inclusion bodies (e.g , Sangiorgi, 1915) For any eosinophil granules to be seriously regarded as specific inclusion bodies, however, they should be demonstrable in the basal layers of the skin, normally free from such particles Further, they should be carefully studied in preparations stained by the Feulgen technique for thymonucleic acid and by the MacCallum test for masked iron, to exclude a nuclear origin, as well as by the oxidase reaction, to exclude a possible derivation from leukocytes Wilson (1937) has demonstrated large granular eosinophil bodies in the malpighian and

basal layers which bear considerable resemblance to Guarnieri, Bollinger, Marchal and other inclusions, but these bodies, before they can be accepted as specific, require further investigation.

As regards nuclear inclusions, it is difficult to reach any definite conclusion Ullmann (1923) described intranuclear inclusions in the cells of a papilloma produced in the vagina of a bitch, which bodies resembled the Lipschütz bodies of herpes Lipschutz has described the presence of both basophilic (1924 *a*) and eosinophilic (1924 *b*) bodies in acuminate and common warts, but these are probably of degenerate nature Wilson (1937) has also found eosinophilic intranuclear inclusions in genital warts

The virus of human warts was first shown to be fitrable through a Berkefeld N candle in 1907 by Ciuffo. This observation has been confirmed with Berkefeld candles of all grades of porosity (Wile and Kingery, 1919, Kingery, 1921; Findlay, 1930).

Reaction to physical and chemical agents. The virus withstands 50 per cent. glycerol (Findlay, 1930) and may, therefore, be preserved in this solution in the refrigerator. It also survives a temperature of 50° C. for half an hour (Templeton, 1935).

IMMUNITY AND TREATMENT

Warts frequently disappear spontaneously, but whether this be due to increase in antibody content of the serum has not been determined Brain (1937) could not demonstrate any antibody in the serum of patients Maderna (1935), however, has shown that complement fixation occurs between wart antigen (made by grinding warts in saline after glycerolization) and the serum of cases of warts.

Findlay (1930) states that he became immune to further infection after three separate inoculations of wart material, so presumably active immunity can be acquired

Maderna (1934) has described a skin reaction of similar type to the Frei test. Wart antigen is injected intradermally and a papulopustular reaction develops

Warts are usually treated by local means directed toward causing them to shrivel up No success has attended the injection of wart vaccines (Brain, 1937) When one wart is destroyed, others may also regress.

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CHAPTER XVI

HERPES ZOSTER (ZONA, SHINGLES)

CLINICAL FEATURES

Predisposing Causes

Although probably the majority of cases of zoster is "idiopathic" or primary, in some a predisposing cause may be found. Thus, secondary zoster occurs after administration of arsenic or bismuth, and in cases of carbon monoxide poisoning, sulfonamides have also been blamed (Attmeyer, 1939). It is also found in cases of pulmonary tuberculosis, malaria, pneumonia, and cerebrospinal fever, it is sometimes related to trauma (see Jadassohn, 1938), and has occurred after the patient has been struck by lightning (Parfitt, 1936). A case has been reported where bilateral zoster of the buttocks followed suboccipital injection of lipiodol (Urechia, 1938); zoster has followed the administration of a spinal anesthetic (Arnold, 1941). In some of these cases it is questionable whether one is not really dealing with a primary zoster occurring by chance in one of the conditions mentioned

Another group of cases developing zoster is that where the spinal cord or column is affected by a disease such as tabes, acute myelitis, spinal tumor, or caries (Kobro, 1940, Mumme, 1941). Zoster may occur in the area of referred pain in cardiac infarction or angina (Parsonnet and Bernstein, 1939, Spillane and White, 1939)

Leukemia, lymphadenoma, or lymphosarcoma may lead to zoster, possibly due to [illegible] ëri *et al*, 1937, [illegible] Poulsen, 1939, [illegible] Catlin, 1946). Marques (1938) has reviewed the literature on the association of zoster and leukemia and finds that it is more frequent in the lymphatic type It may be stated here that both primary and secondary zosters are regarded as being due to the same virus.

Incubation Period

The most reliable information is probably that supplied in various experiments (mentioned below) where the following periods have been found to elapse between the injection of zoster fluid and the development of local vesicles 9 to 12 days (Kundratitz), 7 to 14 days (Bruusgaard), 21 days (Siegl).

Course of the Disease

During the last few days of the incubation period the patient, particularly if young, may be pyrexial, and neuralgia may be experienced in the area which is soon to be affected Various respiratory and digestive disturbances may occur. The rash appears first at the point where the cutaneous nerves come to the surface, and spreads therefrom It has been said that the spread of the lesion occurs distally, along the distribution of the nerve (Stern, 1937). At first the rash is erythematous, but vesiculation soon takes place After 5 to 10 days these vesicles have usually dried up and formed scabs, in very severe cases gangrenous change may occur. Small "aberrant" vesicles may be found in other parts of the body apart from the area mainly involved Some scarring usually remains after the rash has healed Pain, always severe during the rash, may persist for weeks or months, and in elderly persons is specially liable to recur in cold weather In certain cases anesthesia of the affected part may be found

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(*b*) *Endocarditis* A case of cervicothoracic zoster has been recorded which was followed in 7 days by endocarditis (Andréassian, 1934).

(*c*) *Nervous complications.* The central nervous system is probably involved to some extent in most

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Cranial nerve paralysis usually affects the facial nerves, alone or with others (see, e g, Ebstein, 1895, Spota and Alurralde, 1937, Kramer, 1938, Wilson, 1941) Rebattu *et al.* (1933) found facial paralysis in almost 50 per cent. of cases of cephalic zoster, and it was of the Bell or peripheral type Ramsay Hunt suggested that paralysis of the facial nerve associated with an eruption in the external auditory meatus was due to involvement of the geniculate ganglion; Denny-Brown, Adams, and Fitzgerald (1944) reported a case of this type where the geniculate ganglion was quite unaffected, the second cervical ganglion showed inflammation, and there was a motor neuritis of the facial nerve. O'Neill (1945) reviews the question of zoster auris, and concludes that it is not uncommon He found no evidence that Hunt's syndrome is necessarily associated with geniculate ganglionitis, it may be associated with involvement of the trigeminal, second cervical, and vagal ganglion. Tschiassny (1946) also reviews Hunt's syndrome, and concludes that if facial palsy is associated with a facial or occipitocollaris eruption the nerve lesion is not in the geniculate ganglion, if the eruption is on the "geniculate" zone of the external ear, the nerve lesion is to be found in the geniculate ganglion Paralysis of the ocular muscles is said to occur in some 7 per cent. of cases of zoster ophthalmicus (Hewlett, 1906) This condition of paralysis has been recorded by a number of authors, some of whom note its occasional association with hemiplegia (Parkes Weber, 1915, 1916, MacGillivray, 1931; André-Thomas and Buvat, 1931, Rebattu *et al*, 1933, Carmody, 1937, Perrin *et al*, 1938) The internal ocular muscles are usually involved along with the external, recovery occurs in 2 to 3 months

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Meningo-encephalitis, or encephalitis has been described in a number of cases (see Schiff and Brain, 1930, Brain, 1931, Biggart and Fisher, 1938, Gais and Abrahamson, 1939, Parat, Maffei, and Lewi, 1939, Hassin and Rabens, 1944, Gordon and Tucker, 1945, Krumholz and Luhan, 1945)

Schiff and Brain, in an extensive review, mention three possibilities to account for meningo-encephalitis (1) a pure coincidence, as supported by Netter (1930), who reported a number of cases which he considered to be encephalitis lethargica complicating zoster, (2) a pre-existing meningo-encephalitis of undetermined etiology is the primary condition which damages the sensory nerves, thus causing an attack of secondary zoster, (3) zoster virus spreads from the posterior roots and causes meningo-encephalitis In all probability all three possibilities may occur, and

Varieties of Zoster

(*a*) *Bilateral zoster* is uncommon, usually only one side being involved (see Rattner, 1938, Ambler, 1939).

(*b*) *Zoster of the trunk* is the commonest of all types, and probably the thoracic portion is more commonly involved than the abdominal. Hewlett (1906) estimated that 76 per cent. of cases of zoster affect the trunk, and Stern (1937) has found the posterior part to be more extensively involved than the anterior.

(*c*) *Cephalic zoster*. The subject of cephalic zoster has been treated most comprehensively by Rebattu *et al.* (1933), whose paper should be consulted by all interested (for an English summary see *Arch. Neurol. Psychiat.*, 1934, 31, 1101, 1297). Zoster of the head and neck may affect the cervical, facial, buccopharyngeal, ophthalmic, and auricular regions, and certain syndromes are well recognized. Thus, trigeminal zoster affects the distribution of the fifth cranial nerve, and the gasserian ganglion is severely involved Vesicles occur on the forehead, sides of the nose, and face. The cornea may be gravely affected (zoster ophthalmicus) when the nasociliary branch is involved in the inflammatory process. Zoster ophthalmicus presents a variety of appearances such as keratitis, scleritis, and iridocyclitis (see Duke-Elder, 1938, Viallefont, 1939, Edgerton, 1942; Parry and Laszlo, 1943; Neame, 1944). Zoster oticus is manifested by the occurrence of vesicles on the fauces, uvula, or tongue. The preauricular gland is enlarged, and facial and laryngeal paralyses may occur. The condition is extremely painful (see Kelly, 1940; Rosenberger, 1941, Watson, 1941). Various other paralytic manifestations may complicate cephalic zoster, such as lingual paralysis, facial paralysis (*vide infra*), and auditory or vestibular disturbances Ramsay Hunt described the "geniculate syndrome" of facial paralysis, auricular herpes, with or without deafness, tinnitus, and vertigo (see Denny-Brown, Adams and Fitzgerald, 1944), and below.

(*d*) *Genitory-urinary zoster*. Zoster of the external genitalia and the urethra is not uncommon. Zoster of the urinary bladder is, however, extremely rare. Chesterman (1932) recorded a case associated with a typical eruption on the gluteal region, and Rinker (1941) one without skin lesions

(*e*) *Recurrent idiopathic zoster* is also an extreme rarity. Cases have been recorded where a recurrence took place several years after the first attack and usually on the opposite side of the body (Graham-Little, 1937), the same site may be reaffected even within 12 months (Hruszek, 1934)

(*f*) *Zoster generalisatus seu varicellosus* This condition has been described by a number of authors (e g, Fasal, 1909, McEwen, 1920, Parounagian and Goodman, 1923, Levaditi, 1926, Stein, 1928, Davidson, 1934, Hourand, 1938, Barker, 1939, Ferriman, 1939, Fleming, 1939, Seremini, Herrera-Ramos and Isasi, 1939, Campbell, 1941, Wilson, 1941, Director, 1944, Taylor, 1945, Sharpe, 1946). Most of the cases occur in elderly persons in whom, a few days after the ordinary zoster vesicles have appeared, a profuse and widespread vesicular eruption occurs. The case therefore resembles varicella, but the characteristic polymorphism of the lesions of this condition is usually lacking, and serves to distinguish between the two (Hutton, 1935) *These cases frequently give rise to outbreaks of varicella in contacts* and also to other cases of zoster The condition is a borderline one between zoster and varicella and is probably caused by a strain of virus with marked dermotropic properties, tending to resemble closely strains isolated from cases of true varicella, a number of authors regards the condition as being rather a modified varicella than a virulent zoster

Complications of Zoster

(*a*) *Zoster arthritis* The fingers are usually affected, the onset coinciding with that of the eruption Pain and swelling are severe, and movement is greatly limited (Brain, 1931)

(*b*) *Endocarditis*. A case of cervicothoracic zoster has been recorded which was followed in 7 days by endocarditis (Andréassian, 1934)

(*c*) *Nervous complications*. The central nervous system is probably involved to some extent in most cases, for the cerebrospinal fluid regularly shows lymphocytosis and an increase of globulin (Brissaud and Sicard, 1901; Chauffard and Froin, 1902; Schussler, 1935; Gais and Abrahamson, 1939). These changes are more mar

zoster and must be

899). It is of the lower motor neurone type and probably due to involvement of the anterior horn cells. It rarely occurs in zoster of the trunk, although a few cases of muscular paralysis, usually of the abdominal wall, are recorded (e g., by Taylor, 1895, Hewlett, 1906; Söderbergh, 1919, Worster-Drought, 1923; Gais and Abrahamson, 1939)

Zoster of the upper limbs is more commonly associated with muscular paralysis than is zoster of the lower limbs; in fact, paralysis of the lower limbs is exceedingly rare (see Ebstein, 1895, Parkes Weber, 1915, 1916, Chandler, 1935; Wilson, 1941, Parkinson, 1948).

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Meningo-encephalitis, or encephalitis has been described in a number of cases (see Schiff and Brain, 1930, Brain, 1931, Biggart and Fisher, 1938, Gais and Abrahamson, 1939, Parat, Maffei, and Lewi, 1939, Hassin and Rabens, 1944, Gordon and Tucker, 1945, Krumholz and Luhan, 1945)

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Schiff and Brain themselves quote a case of meningo-encephalitis definitely due to the virus of zoster.

Biggart and Fisher's case also suggested that the encephalitis was due to a direct upward spread of the virus, for the autopsy disclosed a dorsal root ganglionitis, a posterior myelitis, and an encephalitis. Perivascular infiltration with lymphocytes occurred in the dorsal half only of the cord, and there was also a diffuse interstitial increase of cells in the posterior horns. Perivascular infiltration was found in the medulla, subcortical cerebellar nuclei, thalamus, hypothalamus, and parietal region.

Krumholz and Luhan found a relatively mild nonpurulent encephalitis of the medulla oblongata, but no infiltrative inflammatory changes in the brain above the medulla. In the cortex, they [illegible] nature of a secondary and "allergic" [illegible] does not occur. The hemiplegia a[illegible]cially the cephalic variety, may be due to a localized thrombosis or hemorrhage produced by the zoster virus (see Gordon and Tucker, 1945).

PATHOGENESIS

The portal of entry of the virus is unknown, and various routes have been suggested. Haslund (1900) pointed out that the disease may be ushered in with upper respiratory symptoms, sore throat, angina, and the like, and suggested, therefore, that the virus enters the body through the tonsils. Thereafter it is carried in the blood stream to one or more spinal ganglia, and in certain cases to the skin also, thus explaining the production of "aberrant" vesicles. Low (1919) suggested that the virus spreads from the nose by the olfactory perineural lymphatics to the meninges and cerebrospinal fluid, and thence to the spinal ganglia.

Neither of these theories explains the specific localization of the virus in particular spinal or other ganglia. To account for this, one of the most striking features in the pathology of the condition (*vide infra*), Montgomery (1921) suggested that the virus enters the nerve endings in the skin and ascends along the nerve sheath in the lymph spaces until it reaches the appropriate ganglion. In our opinion, this theory is the most tenable of the three, and is supported by certain clinical findings. Thus, regional lymphadenitis limited usually to one gland draining the site of eruption is a constant feature, usually disappearing by the seventh day (Raymond and Lebel, 1920, Rebattu *et al.*, 1933). Further, for some days before the appearance of the eruption, neuralgia, hyperesthesia, and paresthesia occur in the region supplied by the nerve, possibly due to some irritant action of the virus ascending therein. While Montgomery suggests that the virus ascends in the nerve, Stern (1937) suggests the reverse. Thus, when in cases of thoracic zoster he injected the appropriate intercostal nerve with alcohol, the skin became anesthetic, but no cessation of the spread of the lesion was observed. Further, in early cases of zoster he found the lesions to spread distally along the course of the nerve. Accordingly he suggested that the eruption was probably caused by some substance traveling slowly down the nerve. He does not indicate, however, how the virus reaches the central position in the first place.

Any theory of the pathogenesis of zoster must endeavor to explain the quite common occurrence of paralytic complications (this subject has been extensively dealt with by Rebattu *et al.*, 1933). The most likely explanation of why these cases occur is that the inflammation in the posterior horns or roots spreads forward to involve the anterior horns or roots. This theory of the central origin of the paralysis does not fit all cases, however, and Montgomery (1921) considers that the paralysis is peripheral. He suggests that the virus enters by the skin, and although usually ascending in a sensory nerve, it may sometimes attack a motor nerve, causing paralysis. Montgomery believes that the reason why paralysis is commonly associated with zoster of the extremities is that the terminal motor nerves are situated so superficially that the virus can enter readily. Paralysis is

said to be more rarely associated with thoracic zoster because here the motor nerves are less easily accessible. The comparative frequency (7 per cent. of cases, Hewlett, 1906) with which cases of zoster ophthalmicus are associated with paralysis of the eye muscles also lends support to Montgomery's theory, as he finds that it is the most superficial nerve, the oculomotor, that is more commonly involved than is the deeper trochlear or abducens. The cases which most definitely suggest a peripheral origin of the paralysis are like those described by Roblin (1920), Rebattu *et al.* (1933), and Masser (1937), who observed facial paralysis associated with cephalic zoster. The paralysis was of the superficial or Bell type, thus suggesting that the virus had attacked the nerve peripherally rather than centrally.

A further point to be discussed concerns the mechanism of production of secondary zoster. In cases where zoster follows on trauma or some infective condition, it seems necessary to postulate that the virus has been latent somewhere in the body and has been stimulated into activity. The site of such latent infection is not known, but perhaps it is in the upper respiratory tract.

In conclusion, we may say that it is probable that the eruption is caused in some way by involvement of the spinal and perhaps certain other ganglia, but as to how the virus reaches these ganglia is unknown. In the present state of knowledge it would be unwise to express an opinion as to whether the spread of infection is centripetal or centrifugal. Montgomery's theory is of interest—that the virus usually ascends sensory nerves to reach the ganglia, and that sometimes it ascends motor nerves, with the production of paralysis.

HISTOLOGY

The vesicle. The histology of the zoster vesicle has been fully described, in particular by Unna (1896), Lipschutz (1925), and McCarthy (1931). The primary change is said to be an affection of the cells in the deeper malpighian layers, some of which proliferate to form giant multinucleated cells. Others undergo marked swelling and form "balloon cells," which stain well only around the periphery, nuclei being seldom discernible. Neighboring areas undergo degeneration, and a unilocular intercellular vesicle results from their fusion. The edematous content of these vesicles increases and forces apart the walls, which are composed of degenerate "balloon cells." Multilocular vesicles may also be found, these are initially intracellular, and form inside "balloon cells," being bounded only by a thin rim of cell wall. The ultimate result is that a multiloculated vesicle is formed by the fusion of a number of these cells.

The fully formed zoster vesicle usually shows both types of change. It is covered over by a layer of horny cells and may rest on the uncovered papillary bodies. If it is of large size the contents of the vesicle may remain serofibrinous with a few polymorphs or else a marked purulent reaction may develop both in the vesicle and surrounding parts. In such cases the dermis shows an inflammatory reaction, being infiltrated with pus cells and lymphocytes.

Inclusion bodies were first described by Lipschutz (1921 *a*, *b*, 1925), but these cannot be found in every case (McCarthy, 1931). They occur intranuclearly, in the epithelial "balloon cells" around the base of the vesicle as well as in connective tissue cells. They are acidophilic and may fill the whole nucleus, but are usually

(1938) have reported the presence of small granular inclusions both in the nucleus and cytoplasm of epithelial cells in the zoster vesicle. These bodies resemble

exactly those reported by the same authors in herpes febrilis (see p. 184). Dahl (1944) has also described EB's.

Healing of the vesicle eventually occurs by crusting, the crust being detached by the growth of subjacent epithelial cells.

The nervous system. The essentially nervous basis of zoster was first recognized by von Barensprung (1862). The classical work on the subject is that of Head and Campbell (1900), who pointed out the uniform involvement of the spinal ganglia and other parts of the primary sensory neurone; they also emphasized the essential pathological unity of both primary and secondary zoster. Usually only one spinal ganglion is affected, showing a marked cellular infiltration, mainly with small mononuclears, both in its capsule and parenchyma. Hemorrhage may also occur with more or less destruction of the ganglionic nerve cells. Healing eventually occurs with secondary gliosis This severe ganglionic lesion naturally affects the other parts of the neurone, the peripheral nerves showing degeneration of those fibers whose nerve cells have been involved in the ganglion, and the posterior nerve roots and columns showing degeneration followed by secondary sclerosis The nerves supplying the affected area of skin may be congested and infiltrated with small round cells (see e g, Charcot, 1889, Marinesco, 1931, Marinesco and Draganesco, 1932, Stern, 1937). Wohlwill (1924) has drawn attention to the posterior poliomyelitis uniformly present in cases of zoster, which is shown by a monocytic infiltration of the posterior horns and even of the bulb and cerebral peduncles Lhermitte and Nicholas (1924) also drew attention to inflammatory, degenerative changes in the posterior horn, and describe similar changes in the anterior horn.

It is evident that herpes zoster cannot be regarded as a mild affection of the skin and spinal ganglia only, as severer nervous changes probably occur in all cases. More recently Denny-Brown, Adams, and Fitzgerald (1944), who examined the spinal ganglia and other portions of the nervous system in 3 cases, drew attention to four histological features distinguishing zoster· (1) ganglionitis marked by pannecrosis of all or part of the ganglion, with or without hemorrhage and surrounded by intense lymphocytic infiltration, and associated with the vesicular eruption in the corresponding cutaneous segment, (2) unilateral myelitis, limited to 2-3 segments, and involving chiefly the posterior horn and root, and dorsal spinal ganglia, some changes are found in anterior horn cells, (3) localized leptomeningitis limited to involved spinal segments and nerve roots, (4) true peripheral mononeuritis, seen not only in the nerves distal to the ganglion but in the anterior nerve root, both within the meninges and in the portion contiguous to the involved spinal ganglion

EPIDEMIOLOGY

As regards sex, males are said to be slightly more commonly affected than females No age period is exempt, but the majority of cases occurs in adults from 20 to 60 (Rivers and Eldridge, 1929 *a*) A case has been described in a 3-day-old infant (Freud, Rook, and Gurian, 1942) Figures have been brought forward to show that the greatest number of cases occurs in the autumn and June (Bokay, 1928) A number of epidemics of zoster has occurred, usually in barracks or schools (see Fuhlrott, 1920, Levaditi, 1926) How the infection is spread from case to case is not known, but as many cases apparently arise spontaneously, or secondarily to some exciting factor, it is probable that the virus may be carried on the skin, in the respiratory passages, or elsewhere in the body. Cases certainly arise in contacts of zoster or varicella, so infection may be spread, presumably, by droplets, direct contact, or fomites It is probable also that human carriers exist (Wooley, 1946)

PROPERTIES OF THE VIRUS

Inoculation Experiments

Human experiments. Zoster fluid injected into the skin of adults will not reproduce the infection (Meineri, 1922). A number of workers has shown, however, that zoster fluid injected intradermally into children under 5 years of age often produces a crop of vesicles Kundratitz (1925 *a*, *b*; Lipschutz and Kundratitz, 1925) was the first to demonstrate this infectivity, which had previously been suggested on purely clinical grounds by Landouzy in 1884. Thus, in one series Kundratitz injected 28 normal children with fluid from 6 cases of zoster, and after an incubation period of 9 to 12 days local papules and vesicles developed in 17 cases, and in 3 of these typical varicella occurred. Numerous other authors have confirmed these findings, both with regard to the production of local lesions and of varicella (see Siegl, 1927, Netter, 1928, who gives a number of references, Cleveland, 1927, Bruusgaard, 1932) The experiments of Bruusgaard are of particular interest. A child was injected with zoster fluid, and 7 days later varicella-like vesicles developed locally. The fluid from these vesicles produced, 14 days after intradermal injection, a generalized varicelliform eruption in another child Further, this child's brother developed typical varicella 14 days later.

It must be noted, however, that Lauda and Stohr (1926) carried out a very extensive series of injections in over 50 children, in no case was a local lesion produced, although 3 of the injected children developed varicella; 3 other children, however, in contact with zoster, but not injected with vesicle fluid, also developed varicella They did not consider that their results demonstrated the infectivity of vesicle fluid derived from zoster lesions.

Animal experiments Lipschutz (1921 *b*) produced a reaction in the scarified corneas of 3 out of 7 rabbits inoculated with zoster fluid, and in 2 of these typical intranuclear inclusions were found Kin (1939 *a*) produced changes in the corneas and testes of rabbits, on inoculation with material from zoster vesicles, inclusions and EB's were found Lesions were produced by intracutaneous inoculation of this material in human beings (1939 *b*) Against this observation has to be set the opinion of a number of investigators that the disease cannot be transmitted to animals (e g, Marinesco, 1922 *a*, *b*, Blanc and Caminopetros, 1922, Marinesco and Draganesco, 1923, Doerr, 1925, Cole and Kuttner, 1925) It may be said that in these various experiments the following animals have been used rabbits, mice, monkeys, cats, sheep, pigeons, dogs, and guinea-pigs In view of these negative results the findings of Eckstein (1933–4) are of interest Eckstein injected monkeys intracerebrally with zoster vesicle fluid, and after 4 to 5 weeks they died with symptoms ir [illegible] perivascular infiltrati [illegible] and medulla. Seconda [illegible] aricella fluid (see p 2 [illegible] iatory changes in the monkey brain

Certain interesting results have been recorded in which, apparently, the virus of herpes febrilis has been isolated from typical zoster vesicles (Luger and Lauda, 1921, 1923–4, 1925, Cipolla, 1923–4, Becuwkes, 1939). The wide distribution of the herpes virus is so well recognized, however, that no particular stress should be laid on this finding

Morphology of the Virus

Elementary bodies of approximately 0 25 μ in diameter can be found in suitably stained films of vesicle fluid (Paschen, 1933, Amies, 1934, Taniguchi *et al*, 1934) These are most numerous 24 to 48 hours after the appearance of the lesion and can be agglutinated by zoster (and varicella) convalescent sera The EB's ap-

pear round, and measure 145 mμ by electron microscopy (Ruska, 1943). Inclusion bodies (already described) have been recorded with regularity only in human lesions, and their precise nature is uncertain. Kin (1940) claimed that his virus passed through a Berkefeld candle.

Cultivation

The virus was found to preserve its activity in tissue cultures of the Maitland type for 4 days, and local skin lesions were produced on intradermal injection of children (Glaubersohn and Barg, 1934). More definite evidence of propagation in tissue culture has not yet been published.

The virus has been found to produce characteristic pocks in the developing chorio-allantois of fertile eggs (Castro-Teixeira, 1936; Kin, 1940). Goodpasture and Anderson (1944) obtained fragments of human skin, infected them with vesicle fluid, and placed them on the chorio-allantois Acidophilic inclusions were found in the nuclei of hyperplastic epithelial cells. The chick membrane itself was not susceptible.

Effects of Physical and Chemical Agents

The virus retains its activity in glycerol in the refrigerator for at least 4 days (Glaubersohn and Barg, 1934).

IMMUNITY REACTIONS

Active Immunity

An attack of zoster is generally regarded as giving a life-long immunity against further recurrence, but such may occur. It is usually believed that varicella seldom occurs in cases that have had zoster, and vice versa, though after some years this cross immunity may disappear. It should be noted, however, that an investigation of schoolboys showed that about 71 per cent of boys with zoster had previously had chickenpox (Medical Research Council, 1938). This figure compared almost exactly with 67 per cent of controls who also had had chickenpox Clearly, these results do not support the view that chickenpox immunizes against zoster.

With regard to serological reactions, *complement fixation* reactions have been demonstrated by a number of authors using zoster antigen and patient's serum (Netter and Urbain, 1924 *a*, 1926, 1931, Netter *et al*, 1924, Bedson and Bland, 1929, Brain, 1933, Hasskó *et al*, 1938). The antigen for these tests consists of either vesicle fluid or dried crusts, the former being probably the best. The reaction is most powerful in the second week of illness, when up to 4 MHD of complement may be fixed Of more interest than this observation, however, has been the demonstration of cross fixation with material from cases of varicella With zoster vesicle fluid as antigen, equally good fixation was obtained in the presence of either zoster or varicella convalescent serum Corresponding results occurred if varicella crusts were used as antigen Zoster antigen does not fix complement in the presence of sera from patients suffering from herpes febrilis (Netter and Urbain, 1924 *b*) Fixation occurs between the sera of cases of arsenical or bismuth zoster and zoster antigen (Netter and Urbain, 1924 *c*) Despite the above positive findings, Thomsen (1934) was unable to demonstrate any fixation between zoster serum and zoster or varicella extract in a large series of cases (83), the converse reaction, with anti-varicella serum and zoster antigen, being also negative The study of Hasskó *et al.* (1938) also rather contradicted the older views Thus, although they found that zoster antigen and zoster serum fixed complement, there was only poor fixation by zoster serum and varicella antigen, or by varicella serum and zoster antigen

[illegible] These reactions were demonstrated with preparations of

[illegible] (Paschen, 1933, Amies, 1934) Zoster

of zoster elementary bodies, but zoster

serum agglutinated varicella elementary bodies to a somewhat lower titer than zoster elementary bodies.

Passive Immunity

*Zoster convalescent serum is said to protect exposed children against the de-*velopment of varicella (Kundratitz, 1925 *b*, Lipschutz and Kundratitz, 1925, Abramson, 1944) Such was not, however, the experience of Lauda and Stohr (1926) who found it devoid of any action in this respect Gundersen (1940) recommended the use of convalescent blood in treatment.

RELATIONSHIP BETWEEN ZOSTER AND VARICELLA

Clinical observations For many years clinical observers have suggested a relationship between zoster and varicella. Probably Bokay in 1888 (von Bokay, 1909) was the first to make the suggestion that zoster and varicella were very closely related, and he continued to support his original view in later publications (e g, 1919, 1924, 1928) Bokay attended numbers of cases where zoster had given rise to varicella in contacts. In certain cases varicella gave rise to zoster in contacts but *probably only one-tenth as commonly as the reverse. In these cases of contact* infection the usual incubation period is from 7 to 21 days, and over half the cases develop 14 to 16 days after exposure to infection

So many authors have published records of varicella occurring in contacts of zoster, and, much less commonly, vice versa, that we can mention only comparatively few by name (Low, 1919, Heard, 1921; Roxburgh, 1923; Roxburgh and Martin, 1926, Wilson and Mitchener-Little, 1926, Netter, 1928, who described 174 examples in France alone, Brain, 1931, Sayers, 1932; Bruusgaard, 1932, Robertson, [illegible] Weissmann-Netter and Lévy, 1938 [illegible] 1938) Thus, during the period of 5 years from 1930–4, 20 outbreaks of varicella were initiated by a case of zoster, the attack rate being 0 97 per cent When an epidemic of varicella was initiated by a case of varicella the attack rate was, however, 3 86 per cent

Varicella in contacts has been recorded after exposure to zoster occurring secondarily to arsenic therapy (Parkes Weber, 1946)

Experimental injections Experimental work performed in recent years has added weight to these clinical observations Thus, Kundratitz and other workers (*vide supra*) showed *that the injection of zoster vesicle fluid produced, in certain* cases, a typical varicellar eruption Kundratitz also noticed that such inoculations failed to "take" in children who had previously had an attack of varicella Further, children inoculated with zoster fluid were immune to later inoculations of varicella fluid

Study of the viruses As regards the two virus agents, neither has been studied very extensively, but sufficient has been learned to show their close relationship *Thus, similar types of elementary and inclusion bodies* have been found in the vesicles, and the histology of the vesicles is practically identical With regard to animals, zoster virus does not appear to be so readily transmissible as varicella It has been shown, however, that following intracerebral injection of zoster or varicella vesicle fluid rhesus monkeys may develop meningo-encephalitis (Eckstein, 1933–4)

Immunological observations Serological methods have yielded proof of the essentially close relationship between the two viruses Thus, as already mentioned, cross reactions between antisera and elementary bodies have been described by Amies, cross complement fixation has also been reported, but as already mentioned, both Thomsen and Hasskó failed to find any evidence of relationship by this latter

technique. With regard to virus neutralizing properties, the findings here are contrary to those obtained by other methods, for Rivers and Eldridge (1929 *b*) found that convalescent zoster serum mixed with varicella virus did not prevent the development of intranuclear inclusions on intratesticular injection of the mixture into monkeys (Rivers, 1926, 1927), whereas convalescent varicella serum and varicella virus did not cause the development of these inclusions

It is generally believed that zoster seldom develops in those who have had varicella, and vice versa. However, certain figures (M.R.C. Report, 1938) do not support this view (see also Ferriman, 1939, Crisp, 1940). Nevertheless, further proof should be adduced before one abandons the common belief that one of these diseases immunizes against the other.

Epidemiological observations. Bokay (1928) examined the incidence of both diseases in Budapest for 10 years (1915–24). He found a striking correspondence in the monthly prevalence of the two diseases, and the peak of the zoster curve regularly occurred a month before that of the varicella curve. Such, however, was not the experience of Rivers and Eldridge (1929 *a*), for with New York statistics they found the zoster figures to fall on a straight line, while the varicella figures were much higher from October to June than from June to September. Dahl (1946) found that the maximum incidence of chickenpox occurred in January-March, whereas that of zoster was usually in August, although sometimes from October to December. Cantor (1921) reported that, while zoster was quite common on the isolated Christmas Island, varicella was unknown. Wooley (1946) made similar observations on Tristan da Cunha, where some cases of zoster failed to give rise to any varicella, although the population had never suffered from this disease and must have been susceptible If the two viruses are closely related, it is admittedly difficult to explain these findings.

Conclusions

It may be said that on the basis of clinical observations and experimental work there is fairly strong evidence for postulating a close relationship between the 2 diseases. That is to say, zoster and chickenpox may be different manifestations of infection by one virus agent (Damm, 1931, Philadelphy and Haslhofer, 1934) It must be admitted that there are certain difficulties in the complete acceptance of this view, which is not held by all (Barrett, 1938).

In the first place, some of the experiments advanced in support of the close relationship of the 2 causal agents have failed to be confirmed by others, and secondly, why does varicella not give rise to zoster so commonly as zoster gives rise to varicella? It is possible that age may explain this, for zoster is commoner among adults and varicella among children (see Dahl, 1946) Thus children coming in contact with zoster are comparatively susceptible, the virus generalizes and causes an attack of varicella, whereas adults coming in contact with varicella are relatively immune, and either the virus produces no disease, or else an attack of zoster, which may be regarded as varicella occurring in a partially immune person That the strains of the causal agents themselves may behave somewhat differently is suggested by the report on school outbreaks already mentioned, in that the epidemics initiated by a case of zoster were much less infectious than those initiated by a varicella case

It is probably wisest, in conclusion, to regard the viruses of zoster and varicella as being closely related strains, the production of the individual syndrome being dependent both on the properties of the particular strain and the tissues of the host

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CHAPTER XVII

HERPES FEBRILIS

HISTORICAL

A virus can be isolated from certain of the herpetic eruptions which is highly pathogenic for experimental animals, and has been studied intensively from this point of view Although herpes febrilis was probably first shown to be infective by Vidal (1873), little interest was taken by bacteriological workers until the end of World War I, when Gruter and Lowenstein reported that herpetic keratitis could be readily produced in rabbits. Soon afterwards, Doerr and his collaborators pointed out that extension of infection to the brain might occur in such animals For the next five years or so a large volume of work was carried out on the virus, mainly by Levaditi and his coworkers in France, and by the Swiss workers, Doerr and his colleagues During these years the chief concern was with the possible identity of the causal agent of herpes with strains isolated from cases of epidemic encephalitis by Levaditi and others Eventually it came to be realized that the two were in fact one and the same virus, although whether this is the cause of epidemic encephalitis in man is still undecided. It should be explained that the term "herpes" by itself refers to the various types of herpetic eruption (e g, febrile and simple) to be described in this chapter, it does not include herpes zoster. The causal agent of herpes is known as the herpes virus, the herpes febrilis virus, or the herpes simplex virus, all three terms are in common use and refer to the same agent.

CLINICAL FEATURES

Herpetic stomatitis It has been established that the common inflammatory condition of the mouth in children, known as aphthous stomatitis, is a manifestation of herpetic infection (Dodd, Johnston, and Buddingh, 1938, Gottron, 1938, Burnet and Lush, 1939 *b*, Burnet and Williams, 1939, Black, 1942, Scott, 1944, Buddingh, 1946, and others to be mentioned) This infection, which should be called acute herpetic stomatitis, is known by a score of other names, for example aphthous, Mikulicz's, Vincent's, or ulcerative stomatitis, acute infective gingivostomatitis, or periadenitis mucosa necrotica Vincent's fusospirochetes may be found in the lesions, but are only secondary invaders.

The diagnosis of these cases has been made by isolating the herpes virus by animal inoculation, and by testing acute and convalescent phase sera for virus neutralizing antibody. The latter tests have shown that herpetic stomatitis occurs in two forms In the primary form, most common in children aged 1 to 6 (newborn infants are resistant), there is a severe general febrile disturbance as well as the local stomatitis The gums are red and swollen Vesicles form on the tongue or buccal mucosa and quickly ulcerate There is oral fetor, and the regional glands are enlarged Lesions may occur also on the lips, face, and fingers The illness usually lasts 10 to 14 days Serological tests show absence of antibody in the acute phase, but its development to high titer during convalescence In this type of case the child is experiencing its first infection with the virus of herpes On recovery, the child is unlikely to develop such a severe illness again The virus persists, however, in the epithelial cells of the mouth or elsewhere, and gives rise to recurrent herpetic vesicles in the mouth or on the lips

In the recurrent form of stomatitis, there is a milder local lesion, without much general disturbance This type of stomatitis recurs throughout life, and sufferers constantly show a high level of virus neutralizing antibody

Primary herpetic stomatitis occurs characteristically in children, but may also

be noted in adults, it is probable that so-called herpetic fever is a primary oral infection (Scott, Steigman, and Convey, 1941). Trench mouth is probably also herpetic in origin. There is no evidence that Vincent's ulceration of the tonsil in adults is herpetic (Steigman and Scott, 1947)

Primary herpetic stomatitis may occur in small outbreaks in families, and is usually spread by an infected adult; the incubation period is about 3 to 5 days

Vaccination with calf lymph is said to benefit herpetic stomatitis (see, e.g, Woodburne, 1941).

Buddingh (1946) has described the syndrome of stomatitis and diarrhea of infants due to an apparently "new" virus, clinically the resemblance to herpetic stomatitis is close (see Ch XVIII).

Herpes febrilis This is the term applied to herpes arising in the course of a pyrexial disease. Thus it occurs commonly in pneumonia, malaria, influenza, and other febrile disorders The usual site is on the lip, when the term "herpes labialis" may be used Other parts of the face may also be involved by the eruption (herpes facialis). Herpetic vesicles are seldom bigger than a large pin-head and develop on a reddened base They are usually filled with clear fluid, but suppuration may occur The commonest site for herpetic vesicles is on the lips, inside and around the mouth, the cheeks, and the auricles

Herpes simplex This term is applied to herpetic eruptions unassociated with pyrexia, which may arise spontaneously, or secondarily to a variety of causes injections such as vaccines, milk, and colloidal metals (see Fischer, 1927, van Rooyen, Rhodes, and Ewing, 1941), ingestion of certain foodstuffs, or following gastric disorder. Herpes has been reported to follow the administration of a general anesthetic (Humphrey and McClelland, 1944) It occurs very commonly after artificial fever therapy, especially after the first exposure, it is less common after second and rare after third exposures (Warren, Carpenter, and Boak, 1940, Keddie, Rees, and Epstein, 1941)

Herpes genitalis Herpetic eruptions are not uncommon on the genitals, being found on the glans or body of the penis, and in the female, on the labia It is seldom that cases in the female are seen by the physician (Sharlit, 1940) Slavin and Gavett (1946 *a*) describe 3 cases of primary ulcerative vulvovaginitis in women

Herpes cornealis Herpetic infection of the cornea may assume a variety of forms, of which the dendritic ulcer is the best known (see Duke-Elder, 1938, Neame, 1942, Hamilton, 1943, Scott, 1944) The inflammatory reaction may be severe and result in serious scarring of the cornea It may be associated with a facial herpes Gallardo (1943) using serological tests, recognized dendritic ulcer to be the primary manifestation of herpetic infection in some cases, in others the infection was evidently recurrent or secondary

Recurrent herpes Certain persons are very susceptible to herpes, and why this should be is not clear It is probable that these people carry the virus in their saliva or elsewhere in the body, Burnet and others (see below) have suggested that in such cases the herpetic infection is contracted in infancy, as an aphthous stomatitis, and persists throughout life Some particular recurring susceptibility of the tissues may allow the virus to exert its pathogenic action from time to time Certainly, recurring genital herpes is most apt to occur in cases of irritation due to chronic gleet and prostatitis (Avit-Scott, 1931) In women recurrent herpes is often associated with the menstrual periods (Blum, 1926) A few cases have been described of recurrent herpes affecting an area of skin previously traumatized (see Findlay and MacCallum, 1940) These observers could not find the virus in the skin between attacks, and tentatively suggested it might remain latent in the spinal ganglia Investigation of this problem of recurrent herpes by means of repeated serological tests has not thrown any light on the matter (Hudson, Cook, and Adair, 1936)

Apart from the question of variations in the host as the predisposing factor in recurrent herpes, it is possible that the strain of virus itself may be one possessed

of unusual properties. Thus, Nicolau and Banciu (1924) inoculated a number of persons with strains of virus from cases of recurrent infection, and found that two of these developed recurrent herpes.

[illegible] gh herpes is usually regarded as a mild local [illegible] may arise from time to time. For example [illegible], 1926) describe *herpès nevralgique de Mauriac,* the symptoms being severe pains, crises of hyperesthesia in the perineum, tenesmus, sphincter spasm, and sciatica, all preceding the appearance of the eruption In less severe cases, herpes may be accompanied by pruritus, backache, and neuralgia. Doubtless these are due to the fact that the virus involves the corresponding gang- [illegible] (see Howard, [illegible]).

[illegible] len onset, rapid course, [illegible] fluid (see Abiteboul, 1936).

According to Janbon, Chaptal, and Labraque-Bordenave (1942), who give a number of references to French papers on herpetic meningitis, the condition may be recurrent; it may follow lumbar puncture, and may be observed as the only manifestation of herpetic infection. They state that meningo-encephalitis can be produced by intrathecal inoculation of the virus in human beings These authors isolated herpes virus from the CSF in one case of herpetic meningitis and refer to another positive result Armstrong (1943) also isolated virus from the CSF, and suggested that herpes may be the causal agent in some cases of "aseptic" meningitis.

Herpes virus has been regarded by some workers as the etiological agent in encephalitis lethargica. This question has not yet been settled, but most authorities are frankly skeptical (see Ch. XCI for a full discussion). Apart from the matter of encephalitis lethargica, there is no doubt that herpes virus may, on occasions, cause a severe and even fatal encephalitis or encephalomyelitis. Thus Warren, Carpenter, and Boak (1940) observed an encephalitis-like syndrome of short duration, and without sequelae, in a group of persons with severe herpes following fever therapy. Smith, Lennette, and Reames (1941) isolated herpes virus from the brain of a child of 4 weeks who died from encephalitis. Histological examination showed a meningo-encephalitis in the pons, medulla, and cerebellum, with areas of necrosis, perivascular cellular infiltration, focal and diffuse inflammation, and nerve cell degeneration with typical herpetic intranuclear inclusions. In another case, diagnosed clinically as equine encephalomyelitis, herpes virus was isolated, herpetic inclusions were found in profusion in glial cells and to lesser degree in neurones in the cerebrum (Zarafonetis *et al*, 1944). Two later cases have been described in young men by Whitman, Wall and Warren (1946). Herpes virus and herpetic inclusions were found in both cases The clinical picture was that of a severe encephalitis Histologically there was leptomeningitis over the cortex, perivascular cuffing in cerebrum, pons and medulla, and marked loss of ganglion cells in the cortex Herpetic inclusions were found in the astrocytes, in sections stained by phloxine-methylene blue. Fisher and Patrick (1947) have described another case.

The cases above described would appear to represent a true herpetic encephalitis, distinct clinically and histologically from encephalitis lethargica. The condition of inclusion encephalitis may here be recalled (see p 000) In this condition, of undetermined etiology, herpetic-type inclusions are found in the central nervous system

THE NATURAL HISTORY OF HERPETIC INFECTION

It has for long been recognized that herpes tends to be a chronic lifelong infection, and that recurrences are stimulated by various factors, especially fever. Burnet, on the basis of his own observations, and those of others referred to elsewhere in this chapter, has advanced the following conception of what he calls the

be noted in adults, it is probable that so-called herpetic fever is a primary oral infection (Scott, Steigman, and Convey, 1941). Trench mouth is probably also herpetic in origin There is no evidence that Vincent's ulceration of the tonsil in adults is herpetic (Steigman and Scott, 1947).

Primary herpetic stomatitis may occur in small outbreaks in families, and is usually spread by an infected adult; the incubation period is about 3 to 5 days

Vaccination with calf lymph is said to benefit herpetic stomatitis (see, e g, Woodburne, 1941).

Buddingh (1946) has described the syndrome of stomatitis and diarrhea of infants due to an apparently "new" virus; clinically the resemblance to herpetic stomatitis is close (see Ch XVIII)

Herpes febrilis This is the term applied to herpes arising in the course of a pyrexial disease. Thus it occurs commonly in pneumonia, malaria, influenza, and other febrile disorders The usual site is on the lip, when the term "herpes labialis" may be used Other parts of the face may also be involved by the eruption (herpes facialis). Herpetic vesicles are seldom bigger than a large pin-head and develop on a reddened base They are usually filled with clear fluid, but suppuration may occur The commonest site for herpetic vesicles is on the lips, inside and around the mouth, the cheeks, and the auricles.

Herpes simplex. This term is applied to herpetic eruptions unassociated with pyrexia, which may arise spontaneously, or secondarily to a variety of causes injections such as vaccines, milk, and colloidal metals (see Fischer, 1927, van Rooyen, Rhodes, and Ewing, 1941), ingestion of certain foodstuffs, or following gastric disorder. Herpes has been reported to follow the administration of a general anesthetic (Humphrey and McClelland, 1944). It occurs very commonly after artificial fever therapy, especially after the first exposure, it is less common after second and rare after third exposures (Warren, Carpenter, and Boak, 1940, Keddie, Rees, and Epstein, 1941)

Herpes genitalis Herpetic eruptions are not uncommon on the genitals, being found on the glans or body of the penis, and in the female, on the labia. It is seldom that cases in the female are seen by the physician (Sharlit, 1940). Slavin and Gavett (1946 *a*) describe 3 cases of primary ulcerative vulvovaginitis in women.

Herpes cornealis Herpetic infection of the cornea may assume a variety of forms, of which the dendritic ulcer is the best known (see Duke-Elder, 1938, Neame, 1942, Hamilton, 1943, Scott, 1944) The inflammatory reaction may be severe and result in serious scarring of the cornea It may be associated with a facial herpes Gallardo (1943) using serological tests, recognized dendritic ulcer to be the primary manifestation of herpetic infection in some cases, in others the infection was evidently recurrent or secondary.

Recurrent herpes Certain persons are very susceptible to herpes, and why this should be is not clear It is probable that these people carry the virus in their saliva or elsewhere in the body, Burnet and others (see below) have suggested that in such cases the herpetic infection is c
and persists throughout life Some p
may allow the virus to exert its pa
recurring genital herpes is most apt to occur in cases of irritation due to chronic gleet and prostatitis (Avit-Scott, 1931) In women recurrent herpes is often associated with the menstrual periods (Blum, 1926) A few cases have been described of recurrent herpes affecting an area of skin previously traumatized (see Findlay and MacCallum, 1940) These observers could not find the virus in the skin between attacks, and tentatively suggested it might remain latent in the spinal ganglia Investigation of this problem of recurrent herpes by means of repeated serological tests has not thrown any light on the matter (Hudson, Cook, and Adair, 1936)

Apart from the question of variations in the host as the predisposing factor in recurrent herpes, it is possible that the strain of virus itself may be one possessed

6 Herpes occurring symptomatically in such varied diseases as cerebrospinal meningitis, pneumonia, diphtheria, influenza, catarrhal jaundice, erythemata, and secondary syphilis yields a virus with the same characteristics as that obtained from cases of primary herpes (Teissier *et al*, 1922 *a*).

7. Herpetic stomatitis yields a virus identifiable as that of herpes by its biological properties A specific serological response occurs in primary infections (see above).

8. Intranuclear inclusion bodies, indistinguishable from one another, have been reported in human herpes febrilis, cornealis, and genitalis, and in experimental lesions in animals (Lipschütz, 1921 *a*, *b*, *c*, Fuchs, see 1933).

THE DISTRIBUTION OF VIRUS IN INFECTED AND HEALTHY PERSONS

1. *Herpetic vesicle*. The virus is abundantly present in the vesicular stage, but when the crust forms all virus has disappeared (Teissier, Gastinel, and Reilly, 1926 *a*).

2. *Blood*. The virus may be found in the blood of persons suffering from herpetic eruptions, although this is probably rare (Löwenstein, 1919, Bastaï and Busacca, 1924 *b*), and has, in fact, been denied altogether (Teissier, Gastinel, and Reilly, 1926 *a*, *b*).

3.

fluid o

in one

patient with no herpetic eruption. This is an important observation and indicates that the virus may, although rarely, be found in the cerebrospinal fluid of patients who, at the moment, do not suffer from herpes. Zurukzoglu (1937) has also isolated two strains of herpes virus from the cerebrospinal fluid of healthy persons.

With regard to the cerebrospinal fluid of herpetic patients, the consensus of opinion is that the virus may be found on occasions (Ravaut and Rabeau, 1921; Bastaï and Busacca, 1924 *a*, Zurukzoglu, 1937). It should be added, however, that a number of competent investigators have been quite unable to detect its presence (e g, Doerr and Zdansky, 1924, Levaditi, 1925, Teissier, Gastinel, and Reilly, 1926 *a*, *b*).

As already described, the virus has been isolated on a few occasions from the CSF and brain of cases of herpetic meningitis and encephalitis

4 *Saliva* Herpes virus has been found in the healthy saliva by a number of workers (e.g, Levaditi, Harvier, and Nicolau, 1921; Doerr and Schnabel, 1921 *a*, Teissier, Gastinel, and Reilly, 1926 *b*) The virus is attached to the salivary epithelial cells and is probably associated with the buccal mucus, as preparations of the salivary glands themselves are avirulent (Levaditi, Harvier, and Nicolau, 1921). The presence of the virus has usually been detected by its power of producing keratitis in rabbits Levaditi and his collaborators have stated that 80 per cent of normal specimens of saliva produce experimental keratitis, but only in 15 per cent. is this keratitis severe enough to be followed by cerebral involvement (*vide infra*). These high figures have not, however, been generally accepted by other workers, who have quite failed to find any virus in normal saliva (e g, Flexner and Amoss, 1925 *c*) Nevertheless there seems no doubt that the virus can on occasions be found in this site

Several workers have investigated the saliva of persons suffering from herpes and have, though rarely, been successful in demonstrating the presence of the virus (e g, Isaicu and Telia, 1922, Flexner and Amoss, 1925 *c*, Nicolau and Poincloux, 1922, 1924) Thus, it has been found in the saliva of patients suffering from labial and facial herpes and herpes of the finger The saliva of persons predisposed to herpetic eruptions contains the virus more frequently than that of other persons, and it may be found in the intervals between attacks (Levaditi, 1926).

"natural history" of herpes (Burnet and Lush, 1939 *b*, Burnet, Lush, and Jackson, 1939 *a*; Burnet and Williams, 1939, Burnet, 1945).

Herpetic infection usually occurs in childhood, often taking the form of an herpetic stomatitis. The virus tends to persist throughout life as a latent infection, giving rise to recurrent herpes of the mouth, lips, genitals, or elsewhere, on appropriate stimulation Primary infection may, however, occur on the skin, vulva, or eye, and may be delayed till adult life (see also Gallardo, 1943; Nagler, 1946, Slavin and Gavett, 1946 *a*).

This concept was formulated on the results of serological tests, as well as skin tests performed by Nagler (1944). Thus, Burnet and Williams found that the sera of most young children contained no antibody, while the sera of most adults gave a positive reaction (see also p. 189). The sera of children with herpetic stomatitis showed no antibody at the time of the illness, but developed it later. Persons with a history of herpes showed virus neutralizing antibody, and reacted to a virus reagent introduced intradermally (Nagler, 1944, 1946). Persons without a history of herpes showed no antibody. When antibody occurred in children's sera it was present to the same high titer as adults. Sera showing a small amount of antibody were seldom found, it being either absent, or present to high titer. Burnet found some evidence that the poorer type of patient was more frequently infected with herpes than one coming from a better house (see also Andrewes and Carmichael, 1930) He concluded that the average public hospital Australian patient was primarily infected between the ages of 1 and 5, and developed stomatitis, the virus persisting throughout life.

RELATIONSHIP TO OTHER SKIN DISEASES

It has been suggested that erythema multiforme is very commonly preceded by herpes (Urbach, 1945)

It appears that certain cases of the skin disease known as Kaposi's varicelliform eruption (see Ch. XXVII), are caused by the virus of herpes (Wenner, 1944, Anderson, 1945, Lynch, 1945, Jaquette, Convey, and Pillsbury, 1946). Wenner isolated the virus from the brain of one case, although it had caused no cerebral signs or lesions A mild and a severe type of herpetic Kaposi's eruption have been described, and the term "systemic herpes" has been used (Barker and Hallinger, 1947). Other reports of isolation of virus from Kaposi's eruption are those of Ruchman, Welsh, and Dodd (1947), and Kipping and Downie (1948)

THE COMMON ETIOLOGY OF HERPETIC ERUPTIONS

To-day all herpetic eruptions (always excepting, of course, those of zoster) are thought to be due to one and the same virus The evidence on which this opinion is based will now be detailed

1. Vesicle fluid from all varieties of herpetic eruption can be inoculated into the human skin with consequent production of a typical crop of "simplex" vesicles (Vidal, 1873, Lipschutz, 1921 *b*, Teissier, Gastinel, and Reilly, 1922 *c*, Frei, 1931, Zurukzoglu and Hruszek, 1933)

2. Vesicle fluid from herpes febrilis produces a keratitis when applied to the rabbit's eye (Lowenstein, 1919, 1920, Gruter, 1920)

3 Herpes cornealis yields a virus with the same characteristic action in animals (Doerr, 1920, Doerr and Vochting, 1920, Luger and Lauda, 1921 *a*)

4 Herpes labialis yields the same virus, which will produce typical herpes cornealis when suitably applied to the human cornea (Doerr, 1920, Doerr and Vochting, 1920, Fuchs, 1921, and see 1933)

5. Vesicle fluid from herpes genitalis also produces keratitis in the rabbit, and man can be reinfected with material taken therefrom (Fontana, 1921, Blanc and Caminopetros, 1921 *c*)

investigated the possible rôle of *Aedes aegypti* as an insect vector, and although unable to come to a definite decision, their work strongly indicated that the strains used had been transmitted thereby.

LABORATORY DIAGNOSIS OF HERPES

Virus can be sought in vesicle fluid, saliva, corneal secretion, or brain suspension When working with brain suspension, it may be advisable to inoculate varying dilutions, as concentrated material may not infect so readily (Fisher and Patrick, 1947).

Films may be stained by Paschen's or other method (see Ch III) and examined for elementary bodies Of more value than this, however, is animal experiment, the simplest procedure being to drop some of the vesicle fluid upon the scarified cornea of a rabbit, and watch for the development of keratitis. If sufficient fluid is available, a rabbit may, in addition, be injected intracerebrally (*vide infra*). If the rabbit does not succumb, its serum should be tested for antibody, as an inapparent infection may have resulted (see Evans, Bolin, and Steves, 1945). Mice, guinea-pigs, and hamsters can also be inoculated intracerebrally and intraperitoneally; the convalescent serum should be tested for antibody (Zarafonetis *et al*, 1944, Evans, Bolin, and Steves, 1945; Whitman, Wall, and Warren, 1946).

A rise in antibody, most readily detected by the neutralization of infectivity for mice or the chorio-allantois, occurs in convalescence from a primary infection. In cases of recurrent herpes, however, it will be difficult to detect an antibody increase owing to the initial high level

PROPERTIES OF STRAINS OF DIFFERENT ORIGIN

Various tests can be adopted in the analysis of herpes virus strains By the use of human and rabbit sera, Slavin and Gavett (1946 *b*) were able to differentiate antigenically between a strain LH isolated from vulvovaginitis and the animal-passed HF strain. Florman and Trader (1947) investigated 3 recent human strains and 1 passed in animals (probably HF) They used corneal challenge experiments in immunized rabbits, and inoculation of mice or the chorio-allantois with virus-serum mixtures, the latter was found the most sensitive, some strain differences could be detected They found that two strains isolated from human nervous disease were not more neurotropic in laboratory animals than a strain isolated from the mouth The mouth strain was as dermotropic on inoculation in the guinea-pig skin as was a strain isolated from nerve tissue. It is evident that herpes virus is pantropic, and the tendency to dermotropic or neurotropic characters depends on the host and not the virus; a strain neurotropic in one host may not show this tendency in another

ANIMAL INOCULATION WITH HERPES VIRUS

A. Rabbits

Routes of injection.

These animals can be infected by a variety of routes

1. *Corneal inoculation*

Following observations of Gruter (1920), Lowenstein (1919, 1920) published the results of corneal inoculation of the rabbit with the contents of herpes febrilis vesicles. He found that a severe keratitis developed and that the infection was transmissible to other rabbits, this observation has been abundantly confirmed After such inoculation the cornea becomes "steamy" in about 10 hours, small vesicles form later, and there is usually an associated conjunctivitis Epithelial cells in the exudate contain small granular inclusions (Blanc and Caminopetros, 1921 *a*).

The virus can be found quite readily in the saliva in cases of herpetic stomatitis (see above).

5. *Conjunctival sac.* The virus can apparently be carried in a more or less inactive condition in the conjunctival sac, but if the cornea is injured, then it may produce keratitis (Grüter, 1924, Busacca, 1925). It may also cause severe unilateral conjunctivitis, developing without obvious cause (Granstrom, 1937).

6 *Other skin lesions.* The virus of herpes has been recovered on rare occasions from zoster vesicles (see p. 161), sycosis pustules (Naegeli and Zurukzoglu, 1935).

HISTOLOGY

(*a*) *The vesicle.* The histology of the herpetic vesicle is similar whatever its site, and was accurately described by Unna in 1896 The vesicle is thick-walled, and arising immediately superficial to a swollen papillary process, is entirely subepithelial in position. The cavity of the vesicle is filled with a fibrinous network and prickle cell debris, while the wall is composed of altered prickle cells showing coagulation necrosis. The most superficial layers of the wall are composed of vesicular cells from which the nuclei have largely disappeared. Superficial to the blister is a layer of almost unaltered prickle cells corresponding to the normal middle layer. The whole lesion is covered over by a thick layer of cells merging at the sides into the granular and superficial prickle cell layers. These cells show coagulation necrosis, the nuclei are pale in color and shadowy in appearance, while the cytoplasm is transformed into a fibrinoid mass. In the vicinity of the vesicle there is some edema of the prickle cell layer and considerable swelling and some congestion of the cutis Healing takes place by a deepening of the process of cornification, and a marked infiltration of polymorphs into the site of the vesicle and surrounding parts.

The lesion of herpes is thus seen to consist of a fibrinous inflammation of the epidermis, which tissue becomes detached, overlying a swollen papilla. The characteristic vesicle forms in the space thus provided between the papillary process and the inflamed epidermis. The vesicle fluid contains polymorphs, large mononuclears, and large epithelial cells sometimes showing in their cytoplasm "minute bodies," staining basophilic and similar to those found in the various experimental lesions of herpes (Da Fano, 1923).

Inclusion bodies were first described by Lipschutz (1921 *a, b, c*) in the lesions of herpes genitalis and febrilis, and occur in the nuclei of those epithelial cells superficial to the vesicle. They are granular, eosinophilic, and measure from 2 μ up to the diameter of the nucleus itself. Lipschutz holds these bodies to represent the virus itself, while Luger and Lauda (1921 *c*) among others believe that they are only products of degeneration (for further details, *vide infra*)

(*b*) *Root ganglia* Howard (1905) demonstrated inflammatory infiltration with polymorphs, and degenerative changes in the corresponding gasserian ganglion in cases of herpes occurring in meningitis and pneumonia.

(*c*) *Cornea.* The changes are those of partial epithelial necrosis and infiltration of the substantia propria with leukocytes Epithelial cells may show typical intranuclear inclusions (Fuchs, see 1933).

TRANSMISSION OF HERPES

From what has been said concerning the distribution of the virus, it will be evident that herpes may be spread by saliva or droplets of oral secretion Primary infection may be contracted directly by kissing, or indirectly, by using infected cups, spoons, and other fomites Infection is most readily transmitted in childhood and the skin and mucous membranes of the young child are probably highly susceptible (Burnet, 1945) Genital herpes is spread by sexual intercourse (Hruszek, 1937, Slavin and Gavett, 1946 *a*), as proved experimentally in the case of the rabbit by Levaditi and Nicolau (1923 *a*). Simmons, Kelser, and Cornell (1933, 1934)

and then right itself only to fall to the other side immediately. Trismus, nystagmus, and excessive salivation may all be found. Excessive lacrimation is a rare symptom (Flexner and Amoss, 1925 *c*). Finally the animal dies quietly with limbs extended. The pathogenesis and pathology of herpetic encephalitis are described below.

"Fixed" virus With intracranial passage the virus becomes exalted in virulence and eventually "fixed," when it shows little cutaneous affinity (Blanc and Caminopetros, 1921 *b*, Le Fèvre de Arric, 1922 *a, b, c*). Such a virus has a very short incubation period and usually kills within 3 days, all the symptoms being greatly exaggerated A well-known strain is the HF of the Rockefeller Institute.

Chronic herpetic encephalitis. Sometimes, after intracranial injection, a chronic type of encephalitis may develop (Da Fano and Perdrau, 1927). This reaction is produced most readily in partially immunized rabbits, but sometimes occurs after primary injections of virus, if the incubation period is prolonged The rabbits suffer from an attack of encephalitis as above described but, while some recover, the majority remain in a semilethargic condition

3 *Intravenous injection*

A number of effects have been reported following injection of rabbits by this route Thus encephalitis and a generalized herpetic eruption were recorded by Doerr and Voechting (1920). Encephalitis is not, however, generally produced unless the brain is first mildly injured, e g, by a gentle knock on the head, or by injection of substances such as urotropine and sodium salicylate which appear in [illegible]

Smith (1931 *a, b*), who also found that these lesions were prevented by immune serum

Myelitis of the lumbar cord may follow intravenous injection, virus reaching that site very quickly and before it can be demonstrated in higher parts of the cord The infection may remain localized to the lumbar region, producing paraplegia, or may ascend in the central nervous system (Koppisch, 1935, Doerr and Hallauer, 1936, Hoff and Potzl, 1937).

Cooke, Hurst, and Swan (1942) found that virus could first be detected in the viscera, where necrotic changes subsequently developed Later, lesions were found in the spinal ganglia and dorsal cord at approximately the same time, finally lesions were found in other parts of the CNS Occasionally the olfactory bulbs might be infective before other regions of the cerebrum Young rabbits usually showed signs sooner than older animals The intracarotid injection of virus in sufficient concentration gave rise to primary encephalitis or myelitis in equal numbers of rabbits The addition of a suspension of toadstool spores in 1 per cent. gelatin, or of mayonnaise, to a concentration of virus, usually noninfective, led almost always to a primary encephalitis, if infection developed.

4 *Dermal inoculation*

This method of inoculation results in a crop of herpetic vesicles in 5 to 7 days. Pustulation may occur before the lesions finally crust over If the skin is tarred prior to inoculation, vesicles develop in 3 to 4 days and are distributed like zoster lesions, the animal frequently succumbs, and the corresponding spinal ganglia contain the virus (Teague and Goodpasture, 1923, 1923–4) Paralytic and trophic lesions may follow injection of the skin of a pad (Teissier, Gastinel, and Reilly, 1929) Flexner (1928 *a*) found that following intradermal injection myelitis occurred with, later, extension to the brain, the lumbar cord contained more virus than the pons

Keratoconjunctivitis may follow the dropping or swabbing of vesicle fluid on to the cornea without scarification (*Gallardo, 1943*).

Soon after Lowenstein's important discovery it was pointed out that nervous symptoms sometimes developed in rabbits with herpetic keratitis (Doerr, 1920, Doerr and Vochting, 1920). These rabbits showed excessive salivation, pyrexia, incoordination, convulsions, trismus and gnashing of teeth, and paresis, all of which indicated an encephalitic process. Death usually occurred in 8 to 15 days, although some survived for much longer. Strains of virus isolated from recurrent herpes are said to produce cerebral involvement (after corneal inoculation) much less commonly than do strains from primary infections (*Lépine and Schoen, 1931*).

Histology of herpetic keratitis The changes are those of an acute inflammatory reaction with considerable congestion of the substantia propria, accompanied by polymorphonuclear infiltration. Main interest centers, however, in the presence of inclusion bodies. These were first described by Lipschutz (1921 *c*) who claimed them to be aggregates of virus These bodies appear even within 7 hours and attain their maximum size within 24 hours, after which they become smaller. They are round or oval structures with a definite edge, being found within the nuclei, usually in the epithelial cells, but less commonly in the fibroblasts. They are eosinophilic and somewhat granular, a clear zone separates them from the hyperchromatic nuclear membrane. Similar bodies have been found by Goodpasture and Teague (1923–4 *a*) who agree with Lipschutz that the finding of these bodies definitely indicates the presence of the virus. Others have observed similar bodies, but believe them to be of degenerate nature (e g, Luger and Lauda, 1921 *a*), the latest work (*vide infra*) supports Lipschutz's original view.

With regard to the cytoplasm, "minute bodies" which are small basophilic granules have been described in epithelial cells by Cowdry and Nicholson (1923) who, however, draw attention to a close resemblance between these and the "Russell bodies" normally found in plasma cells (see Dubreuil and Favre, 1914).

Nicolau (*vida infra*) has described small inclusion bodies which occur in the nuclei and cytoplasm of the cells of the cornea. These bodies are granular and with the progress of the infection fuse to form the larger "Lipschutz" type of body. They closely resemble the bodies described by Da Fano and probably represent the etiological agent

2 *Intracranial injection*

Subdural or intracerebral injection produces an encephalitis, fatal in about 5 days and transmissible to other rabbits, as shown first by Blanc (1921) Under certain conditions a chronic type of encephalitis may develop On occasions intracranial injection may give rise, not to the usual encephalitis, but to a primary myelitis (Doerr and Hallauer, 1936) After repeated cerebral passage, the virus loses some of its affinity for the cornea and does not regularly produce keratitis (Perdrau, 1925) After intracerebral injection of rabbits, the usual incubation period is reduced by 2 days if a second injection (intradermal or intracerebral) is given 48 hours after the original, i e, the second injection has an "aggressin-like" action (Perdrau, 1925)

On intracisternal injection herpes virus tends to produce a meningo-encephalitis, usually acute and fatal, but sometimes chronic and recoverable (Janbon, Chaptal, and Labraque-Bordenave, 1942)

Features of acute herpetic encephalitis Blanc and Caminopetros (1921 *b*) gave one of the first descriptions of this syndrome Following an incubation period of some 4 to 6 days, the animal becomes ill, the temperature rises and remains raised until it falls with the first appearance of symptoms about 2 days later The symptoms are indicative of cerebral irritation, the animal moves about by fits and starts, stopping very suddenly, frequently turns the head round, and breathes irregularly The fore-paws are often lifted and show tremor, the animal may fall to one side,

Pathogenesis of herpetic encephalitis in the rabbit.

Following direct intracranial injection, rabbits die of herpetic encephalitis, moreover when an animal dies of herpetic infection following injection by other routes, it is also found to present evidences of involvement of the central nervous system.

It was at first thought that when encephalitis followed a peripheral lesion such as keratitis the infection spread by the blood stream, because Doerr and Vochting (1920) were able to produce encephalitis by intravenous injection. Levaditi (1922) claimed, however, that the virus spread from the cornea by the retina and optic nerve. The extensive work of Goodpasture and Teague (1922–3, 1923–4 *b*) has also shown that the virus usually spreads to the brain from peripheral lesions by the nerves and not by the blood (see also Nicolau, Dimancesco-Nicolau, and Galloway, 1929) In the case of the cornea, for instance, the virus spreads by infecting the axis cylinders of the branches of the fifth cranial nerve, and the first intracranial lesion produced is in that part of the pons and medulla corresponding to the central connection of this nerve. Goodpasture and Teague further showed that the virus can spread to the brain along the vagus, following injection of the tracheal mucosa, along motor nerves after injection of the masseter, and along sympathetic nerves after intrahepatic inoculation In all these cases the first parts of the brain to be affected are those corresponding to the central connections of the affected nerves

It is possible that corneally inoculated virus may pass to the olfactory mucosa by the nasolacrimal duct, and thence invade the brain via the olfactory bulbs (Gaviati, 1922)

Goodpasture (1925 *a*) has shown that when herpetic encephalitis develops in rabbits after contact with infected animals the virus reaches the central nervous system through the sensory divisions of the fifth and ninth nerves Further, histological changes are first noted in association with the central nuclei of these nerves He has inferred, therefore, that in these cases the portal of entry is by the mucosa of the mouth, nose, or throat.

McClellan and Goodpasture (1924) employed an ingenious method for detecting the site of damage in these various experiments. They injected trypan blue intravenously just before death, immediately after death the brain was fixed by intracarotid injection of formalin and was prepared by Spalteholz's method The affected area of the brain appeared bright blue

Marinesco and Draganesco (1932) have also published studies which strongly support the theory that the virus reaches the central nervous system by the nerve tracts, for after corneal inoculation they found the first lesions to develop around the central connections of the fifth nerve, the reaction being meningeal and most marked on the inoculated side Examination of the bulb showed a destruction of the nerve fibers in the descending trigeminal root.

After nasal instillation these authors recorded lesions in the olfactory bulbs, and changes in Ammon's horn They also injected the vagosympathetic trunk, obtaining central lesions in the dorsal vagus nucleus, descending root of the trigeminal, and spinocerebellar fibers Inoculations were also made into the facial nerve, and into the masseter muscle, following this the gasserian ganglion showed intense infiltrative changes and lesions of the central nervous system were also found After intrasciatic injection lesions were produced in the nerve and the cord (mainly on the inoculated side), these were inflammatory and took the form of interstitial neuritis and ganglioradiculitis With the progress of the infection lesions were also found in the cord and bulb

Two further experiments by these workers may be quoted to emphasize the experimental basis for believing that herpes virus spreads by the nerves After transection of a sciatic nerve, injection of the distal portion produced no encepha-

5. Other routes.

Nasal instillation secures infection, and virus spreads by the trigeminal nerves (Gordon, 1928).

Rabbits can be infected subcutaneously and by intramuscular injections. Injection into the anterior chamber also gives positive results, as do injections by the following routes: trachea, conjunctiva, retina, buccal mucosa, ovary, or testicle (Goodpasture and Teague, 1923–4 *a*); external ear (Vieuchange, 1936); prepuce or vagina (Levaditi and Nicolau, 1923 *a*), and sciatic nerve (Pette, 1926, Teissier, Gastinel, and Reilly, 1929). Rabbits are susceptible to contact infection from cases of herpetic keratitis (Goodpasture, 1925 *a*).

Blood changes in infected rabbits. There is a polymorphonuclear leukocytosis with relative lymphopenia, and the count may rise to 17,000 or even 25,000 per c.mm Red cells are not affected (Le Fèvre de Arric, 1923; Remlinger and Bailly, 1926 *b*).

The distribution of virus in the infected rabbit.

The ocular secretion, after corneal inoculation, may contain the virus for as long as 7 days, and it can be most readily recovered from the nictitating membrane (Scott, Steigman, and Convey, 1941).

The blood may sometimes contain the virus (Luger, Lauda, and Silverstern, 1921, Greenbaum and Harkins, 1925, Remlinger and Bailly, 1926 *b*, Gildemeister and Heuer, 1929).

The cerebrospinal fluid is frequently virulent (Urbain and Schaefer, 1927, Flexner and Amoss, 1925 *c*; Gildemeister and Watanabe, 1930–1) Flexner and Amoss have shown that the virus is most frequently found in the cerebrospinal fluid in cases where meningitis is a prominent feature, such as after intracranial injection of unfiltered virus-containing material From 1 to 3 days after inoculation by this route the virus may be found in the cerebrospinal fluid It then seems to disappear for a short time only to reappear, concurrently with pleocytosis, from the fifth to sixth day onwards (Nicolau and Kopciowska, 1931).

The central nervous system Virus can usually be recovered with ease from the brain of rabbits dying of acute herpetic encephalitis, especially after glycerolization In animals dead from chronic herpetic encephalitis, treatment of the brain with glycerol or alumina may be needed to "unmask" the virus (Da Fano and Perdrau, 1927, Perdrau, 1938). Perdrau (1938) found that virus could be recovered directly from the brains of rabbits which developed encephalitis 6 months after active immunization with living virus Good (1947) recovered virus after 9 months from the brain of a rabbit that developed an acute illness on 3 occasions, induced by anaphylactic shock

French authors have described the condition of autosterilizing neuroinfection, certain rabbits injected with strains of herpes die, apparently from encephalitis, but no virus can be isolated from their brains (see Lépine and Schoen, 1931) It is claimed that the reaction of the tissues has destroyed the virus This question is further discussed in the chapter on encephalitis lethargica (Ch XCII)

Peripheral nerves After intracranial injection the virus may be found in the peripheral nerves in a certain percentage of cases (Nicolau and Dimancesco-Nicolau, 1928, Nicolau, Dimancesco-Nicolau, and Galloway, 1929, Kopciowska and Stroian, 1929).

Secretions The saliva and nasal secretion may harbor the virus (Perdrau, 1925, Urbain and Schaefer, 1927), as may the urine and feces (Flexner and Amoss, 1925 *c*, Urbain and Schaefer, 1927)

Viscera The spleen and suprarenal may contain the virus (Luger, Lauda, and Silverstern, 1921, Urbain and Schaefer, 1927) The following organs do not contain virus lung, kidney, ovary, testes, striped muscle, and skin (Urbain and Schaefer, 1927)

as well as lying free. They are stained black by osmic acid, give a positive MacCallum test for masked iron, and are almost certainly nuclear in origin (Cowdry and Nicholson, 1923). Indeed they resemble rather closely the Gamna-Favre bodies of lymphogranuloma inguinale.

3. "Minute bodies" measure 0 3 μ to 0 5 μ in their longest diameter and are either bacillary or coccal in shape. They are found in the cytoplasm of neuroglial cells and macrophages but may also lie free. These bodies do not react to Mac-
...dria (Cowdry
...l by Da Fano
...alitis (1921).
...ose described
...and nucleus
of nerve and glial cells, also sometimes in vascular endothelial and mononuclear cells

Chronic lesions. Da Fano and Perdrau (1927) obtained a chronic meningo-encephalitis in partially immunized rabbits The animals developed a typical herpetic syndrome from which they recovered, either completely or with lethargic and apathetic symptoms. The histological findings were as follows: widespread chronic cellular infiltration, extensive destruction of nerve cells; true- and pseudo-calcification, and formation of young scar tissue. The brains of such rabbits were not infective on removal but became so after preservation in glycerol Perdrau (1938) found that alumina would also activate virus. Histologically, such changes resembled those of human encephalitis more than did the acuter lesions.

(*b*) *Following injection by other routes.*

The appearances of encephalitis following corneal and dermal inoculation are similar to those just described, except that the meningeal changes are usually less marked The lesions are usually most pronounced in the area corresponding to the central connections of the nerve supply to the site of inoculation, as has been emphasized by Goodpasture and Teague, and by Marinesco and Draganesco. When virus is injected intravenously, the lesions are diffuse from the start.

(*c*) *Other factors governing the histological appearances.*

1 The meningeal reaction is more evident with an inoculum of unfiltered brain tissue than with a Berkefeld filtrate (Flexner and Amoss, 1925 *c*).

2. The virulence of the strain also plays a part Animals injected with a strain of herpes virulent for nervous tissue show the characteristic reaction above described Animals injected, however, with a strain relatively avirulent for nervous tissue show a much more chronic type of reaction, the meningitis is mainly mononuclear, parenchymatous changes being slight and mainly restricted to perivascular cuffing (Le Fèvre de Arric, 1922 *b*, Flexner and Amoss, 1925 *c*; Goodpasture, 1925 *b*).

3 Attention must here be drawn to the description given by McCartney (1924) and others that in a proportion of stock animals, prior to intracerebral injection altogether, may be found perivascular and subependymal mononuclear infiltrations due to *Encephalitozoon cuniculi* It would appear, therefore, as though the description of certain authors, particularly with regard to the more chronic lesions (e g, Le Fèvre de Arric, 1922 *b*, Flexner and Amoss, 1925 *c*), should be accepted with some reserve

4 Very young rabbits are said to be strongly resistant to herpetic encephalitis (Goodpasture, 1925 *b*).

B. Guinea-pigs

1. *Corneal inoculation* with rabbit-passed virus produces keratitis (Doerr and Vochting, 1920, Blanc and Caminopetros, 1921 *a*), but spread to the brain apparently does not occur (Doerr and Schnabel, 1921 *b*)

litis. If, however, the nerve was crushed, and an injection made below the site, then infection eventuated. As the authors stated that the nerve fibers were destroyed by crushing, they claimed that the infective agent must have traveled centripetally in the interstitial spaces, and not along the axis cylinders Further, as meningitis is such a noticeable feature of herpetic nervous lesions, it is suggested that the virus travels in the interstitial and perineural lymphatics to reach the cerebrospinal fluid and thus penetrate to the nervous parenchyma.

Levaditi and his coworkers have investigated the fate of virus instilled nasally. They found that it usually spreads by the trigeminal route, and can first be detected in the gasserian ganglion. Although lesions may be found in the olfactory bulbs, this cannot be an important source of spread, for surgical extirpation fails to prevent encephalitis (Levaditi and Haber, 1935; Levaditi, Hornus, and Haber, 1935).

Whether the virus spreads by the axis cylinders or by perineural lymphatics is not known, but nerves are evidently the important pathways of infection Blood-spread may, however, play some part, for intravenous injection may give rise to herpetic encephalitis or myelitis, the former especially after cerebral trauma It may be mentioned that in one of Marinesco and Draganesco's experiments virus was injected into the peripheral extremity of a cut sciatic nerve Contrary to expectation, herpetic encephalitis developed, almost certainly due to blood-spread

The histology of herpetic encephalitis in the rabbit.

(*a*) *Following intracranial injection.*

Acute lesions. Within 24 hours of injection changes are found in the neighborhood of the needle track, where there is hemorrhage. An edematous area in which the vessels show cuffing with polymorphs, some lymphocytes and plasma cells, surrounds this hemorrhage Some basal hemorrhages are also found. With the progress of the infection the following changes occur. The meninges, mainly those of the base, show considerable infiltration with polymorphs, small lymphocytes, large mononuclears, and plasma cells, and even at an early stage many of the polymorphs are necrotic, there is also proliferation of the fixed cells of the meninges The vessels penetrating the brain show perivascular cuffing with cells similar to those infiltrating the meninges, and there is hypertrophy and multiplication of the lining endothelium The adventitial cells also proliferate, while the parenchyma of the brain shows some vacuolation and destruction of nerve cells in the neighborhood of the injection, and there are also lymphocytic infiltrations and subependymal neuroglial proliferation Such, then, are the appearances within the first 4 days With the further progress of the infection considerable hemorrhage occurs in the parenchyma, and there is destruction of nerve cells The polymorphs become largely necrotic, and pyknotic debris is found in the meninges. The cellular infiltrations in the meninges, and around the vessels, are now composed mainly of lymphocytes, mononuclears, plasma cells, erythrocytes, and fibroblasts, with some few remaining polymorphs In animals surviving for some time the main lesions are meningeal and perivascular infiltrations with small lymphocytes The optic thalamus, midbrain, and pons show a particular tendency to be attacked (Da Fano, 1923).

Inclusion bodies A number of different types of inclusion body has been investigated experimentally by Da Fano, Cowdry, and Nicholson, and others

1. Nuclear inclusions are either small basophilic granules (Da Fano, 1923), or larger oxyphilic bodies—*neurocorpuscules encephalitiques* (Levaditi and Harvier, 1920 *a*, Levaditi, Harvier, and Nicolau, 1922) Both of these are probably degenerative products of chromatin produced by virus action.

2 Large extra- and intra-cellular bodies are rounded and acidophilic, varying from 0 6 μ to 5 μ in diameter, found mainly in endothelial and mononuclear cells,

Certain chemicals introduced into the skin prior to an intracutaneous inoculation were found to enhance or retard the development of the infection (Olitsky and Schlesinger, 1941).

After intravenous inoculation, a primary myelitis may develop (Doerr and Hallauer, 1936).

Young animals have been shown to be more susceptible to the extraneural inoculation of virus than older ones (Lennette and Koprowski, 1944); in agreement with this principle it has been found that suckling mice are readily infected by the nasal route (Berry and Slavin, 1943). Virus was found to pass to the brain by the trigeminal and olfactory pathways (Slavin and Berry, 1943 *a*), and it could also be found in the lungs due to direct aspiration, virus was also found to disseminate by the blood to reach the liver, spleen, and less commonly the adrenals and marrow, inclusions could be found in the lymph nodes draining infected organs (Slavin and Berry, 1943 *b*).

Young (2-week) mice can also be infected by intracutaneous inoculation and scarification of the pad, and experiments on the method of spread have been carried out by Evans, Slavin, and Berry (1946) They proved that if the inoculated foot was amputated after 2 hours, the CNS was not invaded by virus passing along nerves, *some animals died of encephalitis, however, virus reaching the brain from* metastatic foci in the viscera, these organs became infected by blood or lymph spread Amputation after 24 hours had no effect, animals dying with the paraplegia characteristic of infection reaching the CNS by nerve paths direct from the foot.

Infected mice were more susceptible to the effect of glucose injected intraperitoneally than normal animals (Hoyt, Holden, and Rawson, 1939).

The administration of insulin or metrazol before the intraperitoneal or intracerebral injection of virus had no influence on the result, metrazol given after an intraperitoneal injection did not facilitate the passage of virus to the CNS (Holden, 1940).

D. Rats

Rats can be infected by corneal inoculation (Teissier, Gastinel, and Reilly, ... ss, 1925 *b*; Smith, Lennette, and Reames, ... on may give rise to a primary myelitis ... in vitamins B_1 and B_2 are not definitely ... than healthy rats (Cowdry, Lucas, and Neff, 1935).

E. Monkeys

M rhesus cannot be infected with herpes, but callithrix monkeys can be infected by intradermal inoculation (Teissier, Gastinel, and Reilly, 1925) Crusted papules or vesicles appear but the infection remains strictly dermotropic without generalization Callithrix monkeys can be infected after several intracranial injections (Lépine, 1929, Levaditi and Lépine, 1929) Cebus monkeys, after intracerebral injection, may develop a chronic type of encephalitis resembling the human disease, both clinically and histologically (Zinsser, 1929, McKinley and Douglas, 1930) Lesions resembling human encephalitis have also been produced in chimpanzees (Delorme, 1929) *M synicus* and *M cynomolgus* can also be infected (Blanc and Caminopetros, 1921 *a*)

F. Cats

These animals are susceptible to intracerebral injection (Remlinger and Bailly, 1925, 1926 *c*, Urbain and Schaefer, 1929) Doerr and Schnabel (1921 *b*) were unable, however, to infect by this route.

2. *Intracranial injection* gives rise to an encephalitis (Blanc and Caminopetros, 1921 *c*). The virus was said to lose its pathogenicity for the rabbit after intracranial passage (Dimitrieff, 1926, Rose and Walthard, 1925–6). Flexner (1928 *b*), attempting to repeat this work, came to the conclusion that these results had only been achieved because a strain of low virulence had been used. Olitsky and Long (1928) carried out experiments with the weak encephalitic strain "C," and found that when injected intracerebrally no infection developed By means of a special double injection intracerebrally and corneally, however, the infection was transmitted

3. *Intravenous injection* may produce primary myelitis (Doerr and Hallauer, 1936).

4. *Dermal injection.* After tarring the skin a zoster-like lesion may develop (Teague and Goodpasture, 1923–4), but lesions can be produced by simple scarification (Bedson and Bland, 1928).

5. *Pad injection* Various workers have shown the pad of the foot to be susceptible (e g, Gildemeister and Herzberg, 1925; Bedson, Maitland, and Burbury, 1927). When inoculated with human vesicle fluid the sole of the foot appears hot, red, and swollen in two days, and by the third day vesicles appear, and soon coalesce. Alternatively, after rubbing virus into the scarified skin, vesicles appear by the second day. Although recovery is the rule, generalization may occur (Bedson and Crawford, 1927, Teissier, Gastinel, and Reilly, 1929; Urbain and Schaefer, 1929). After 4 to 8 days paralysis of the affected paw develops, and paraplegia with complete paralysis may occur. After injection of the pad, Nicolau bodies (see above) may be found in the malpighian cells.

6. *Intragastric injection* After introduction of virus into the stomach, infection results (Urbain and Schaefer, 1929).

C. Mice

Mice can be infected intracranially (e.g, Blanc and Caminopetros, 1921 *c*, Andervont, 1929, *a*, *b*, Fischl and Schaefer, 1929, Schaefer, 1930, Gildemeister and Ahlfeld, 1936, 1937, Burnet and Lush, 1939 *c*).

Steigman and Scott (1942) have described a method for the rapid adaptation of human strains to the mouse brain Rabbits are inoculated on the cornea, and when the infection is established, the nictitating membrane is removed aseptically, ground, and a suspension inoculated intracerebrally in mice Signs of infection usually take up to 10 days to develop, but on passage the incubation is shortened to 3–4 days

Herpes strains can be titrated by intracerebral inoculation (Levaditi and Bonét-Maury, 1941) The titer can be expressed by calculation of the LD_{50} (Bonét-Maury, 1941). It is important to realize that some of the mice that fail to die in titration experiments may have contracted a subclinical attack, and should be challenged by a subsequent injection of virus before concluding that they are not infected (Levaditi, 1941 *b*)

Mice can also be infected more or less readily (according to the strain of virus) by pad, corneal, intra-ocular, intracutaneous, subcutaneous, intraperitoneal, and intravenous inoculations, and by nasal instillation (e g, Andervont, 1929 *b*, Gildemeister and Ahlfeld, 1936, 1937, Burnet and Lush, 1939 *c*, Findlay and MacCallum, 1940) Certain special observations will now be mentioned

Following inoculation into the vitreous, it was found that virus spread by the trigeminal nerve caused lesions in the gasserian ganglion and nucleus of the fifth nerve, with the development of encephalitis (King, 1940)

The HF strain was found to be very invasive on intraperitoneal inoculation, but less infective on intranasal instillation, while it failed to produce infection by the subcutaneous route (King, 1940) It is generally agreed that the subcutaneous route is one of the least effective.

usually granular, body often filling the whole nucleus, although smaller forms are described. The bodies have been repeatedly found in human and in all kinds of experimental animal lesions After local injections of virus into the retina, various mucosae, adrenals, liver, ovary, etc, Goodpasture and Teague (1923–4 *a*) came to the conclusion that the presence of these bodies was always associated with that of the virus The nature of these bodies has been investigated in different experiments

1. Cowdry (1930) studied the inclusions as found in the testicles of infected rabbits, and compared them with somewhat similar inclusions found in virus "III" infection The most important results of this work were that the bodies were found to be resistant to fixative, indicating a protein constitution, and they did not give a positive Feulgen reaction, suggesting that they were not composed of chromatin

2. The development of these structures has also been studied in tissue culture Rivers *et al.* (1929) grew cornea by the tube technique and noted the development of typical Lipschutz bodies in virus-infected specimens Andrewes (1930) found Lipschutz bodies to develop regularly in infected cultures, and claimed that immune serum would prevent their occurrence Both Lipschutz bodies and granules which may possibly be the "minute bodies" of Da Fano have been found in the chorio-allantois of infected fertile eggs (Dawson, 1933) Inclusions develop in human chorion and amnion grafted on the chorio-allantois Anderson (1940) found inclusions in many different types of cell in the infected embryo

3. Micromanipulation studies have been recorded (Baumgartner, 1935 *a* and *b*) which would appear to indicate quite definitely that these bodies contain the living virus, for herpetic keratitis has been reproduced by a single washed inclusion

4. Micro-incineration studies (Rector and Rector, 1933) have shown these structures, when in an early stage, to contain considerable quantities of inorganic material which becomes progressively less as the body ages The mature herpetic intranuclear body may contain no inorganic matter, thus differing markedly from intracytoplasmic bodies in which inorganic material is quite abundantly present.

5. The ultracentrifuge has been utilized by Lucas and Herrmann (1935), and it has been shown that the intranuclear body and the chromatin have different properties. The body is lighter than the chromatin and passes to the opposite pole of the nucleus, sometimes chromatin has to pass through the substance of the body, and if so is found to be separated from it by a distinct space These facts make it unlikely that the body is composed of chromatin

6. Vaccinia and herpes can both grow in individual rabbit corneal cells and produce their characteristic inclusions (Syverton and Berry, 1947).

In conclusion it may be said that opinion is tending more and more to uphold the original view of Lipschutz that the bodies described by him are structures consisting of the living virus, and not degenerative material derived from chromatin. It is possible that Nicolau's bodies represent the initial, and the Lipschutz body the later, stages of growth

Intracellular position of herpes virus. The organism would appear to occupy an intracellular position in infected tissue, such as brain If such tissue is homogenized, that is to say, so treated mechanically that every cell is ruptured and its normal structure destroyed, the resultant product is very much more active than the same material before homogenization (Buggs and Green, 1936). The virus is probably not, however, very firmly attached to the cells as it has been shown to be freely diffusible from infected brain Thus, if infected tissue be placed in glycerol along with normal brain, subsequent tests show that the virus leaves the infected brain to enter both the glycerol and the normal tissue (Remlinger and Bailly, 1926 *a*). The results of experiments by Perdrau and Todd (1936) also support the intracellular position if virus is injected in a testis and suspensions thereof are prepared, methylene blue does not destroy the virus in the presence of light after 18 hours, but virus is destroyed if the suspension is prepared earlier

G. Dogs

Dogs are susceptible only to intracerebral injections of virus which has been passed through cats (Remlinger and Bailly, 1926 *d*, Urbain and Schaefer, 1929).

H. Other Susceptible Animals

The following animals have been used. pigeons, geese, and hedgehogs (Remlinger and Bailly, 1927); porcupines (Remlinger and Bailly, 1931); and foxes (Levaditi, Lépine, and Schoen, 1929).

Cotton rats can be infected cerebrally and nasally (Florman and Trader, 1947). Hamsters are very susceptible to intracerebral and less so to intraperitoneal inoculation (Wenner, 1944; Zarafonetis *et al*, 1944).

I. Simultaneous Injection of Herpes and Rabies

If mice are injected intracerebrally with street virus and herpes, and the brain passed in series, rabies virus is quickly suppressed and only herpes is passed (Levaditi, 1942 *a*).

MORPHOLOGY OF HERPES VIRUS

Elementary bodies have been described by Taniguchi *et al.* (1934). These bodies can be stained by Giemsa's and other methods (see Ch. III), and by direct micrometry appear to be about 0 25 μ in diameter. They have been found in the vesicular fluid of early cases of herpes labialis, and have also been demonstrated [illegible] ing free. [illegible] nan her-

The size of the virus particles has been estimated by means of Elford's gradocol membranes, Elford (1933) and Elford *et al.* (1933) giving the size as from 0.1 μ to 0.15 μ, while 0.1 μ to 0 3 μ are the limits defined by Levaditi *et al.* (1936) The figure varies according to the source of the virus and the animals used to test the filtrates (Levaditi, 1941 *a*) By centrifugation the size has been calculated to fall *between 0.18 μ and 0 22 μ (Bechhold and Schlesinger, 1933).*

Inclusion bodies have already been described and only 3 varieties are worthy of further discussion. First, the "minute bodies" described by Da Fano (1923) and other workers, secondly, Nicolau bodies, and thirdly, the larger bodies of Lipschutz (1921 *a, b, c*).

(*a*) The "minute bodies" occur in the cytoplasm of infected cells in experimental encephalitis of the rabbit and usually stain as basophilic granules. The investigations of Cowdry and Nicholson (1923) have shown that these bodies are unlikely to be of nuclear or mitochondrial origin. Judgment must, however, be reserved as *they have not been found commonly.*

(*b*) Nicolau aroused considerable interest by reporting the presence of small granular inclusions in infected tissues of the rabbit and guinea-pig (Nicolau, 1937, Nicolau and Kopciowska, 1937, 1938) These bodies stain, usually blue, by Nicolau's oxalated methyl-blue-acid-fuchsin method, they measure from 0 1 μ to 0.5 μ in diameter and resemble typical elementary bodies as they appear when inside cells The bodies occur in the cytoplasm and nucleus, both of neuronal and neuroglial cells in the rabbit's brain They also occur in the malpighian cells after pad injection in the guinea-pig After neural injection of rabbits they can be found in and between the nerve fibers

With the progress of the infection the granules may fuse to give larger eosinophilic structures in the cytoplasm or nucleus These nuclear inclusions resemble Lipschutz bodies, while the smaller cytoplasmic granules resemble Da Fano bodies

(*c*) *The Lipschutz body.* This has undoubtedly the strongest claim to be regarded as the typical inclusion body of herpes febrilis It consists of an eosinophilic,

The histological changes occurring in the infected embryo have been particularly studied by Anderson (1940). After inoculation of the chorio-allantois, small plaques were found in 48–72 hours, with inclusions in ectoderm and mesoderm These cells proliferated, and there was a mild inflammatory reaction. Ectodermal cells underwent necrosis and desquamation On serial passage, the severity of the lesions increased and they became more destructive than proliferative; petechial hemorrhages and thromboses in small vessels were found. The membranes of older embryos were more resistant than those of younger.

On amniotic inoculation, Anderson found inclusions in the amniotic ectoderm, mesoderm, and muscle cells, in the embryonic skin and pharyngeal epithelium. When virus was inoculated by scarification of the skin, there were necrotic changes in the epithelium, and nuclear inclusions were found also in striated muscle. On

multiplication. The epithelial cells proliferate, and undergo necrosis This mode of spread was found on chorio-allantoic and amniotic inoculation

(2) Virus invaded the blood stream at the site of inoculation, and was disseminated, causing a generalized infection Inclusions were found in the endothelium of larger vessels and capillaries. On successive membranal passage, the virus produced more severe lesions in the endothelium, and mesoderm such as cardiac muscle and liver; inclusions occurred in these sites and also in interstitial tissue in the lungs, spleen, and subserosa of the gut, glomerulonephritis might be seen. These metastatic lesions could be prevented by the intravenous administration of antiserum

(3) Virus might spread by nerve pathways Following subcutaneous injection, an ascending myelitis might result, and after intracerebral inoculation a descending encephalomyelitis.

Reaction to Physical Agents

Heat. 56° C for ½ hour, or 37° C for 24 hours, destroys the virus (Löwenstein, 1919, 1920) The thermal inactivation time of two stock strains was 80 hours at 41 5° C., a recently isolated strain was destroyed in 50 hours at this temperature. The neurotropic factor of these strains was more resistant to heat than the dermotropic (Boak, Carpenter, and Warren, 1940). Radiothermy (107° C for 1½ hours) failed to cure herpetic encephalitis of rabbits (Kopeloff and Holden, 1931–2)

Ultrapressure The virus is destroyed by a pressure of 3,000 atmospheres (Basset *et al*, 1935)

Centrifugation. Bedson (1927) showed that the virus could be deposited at 5,000 r p m in 2 to 2½ hours

Electrical reactions and effect of pH The iso-electric point is between 7.2 and 7 6 (Bedson and Bland, 1929 *a*) and the virus particles are negatively charged at pH 7 8 to 6 8 (Nicolau and Kopciowska, 1930 *a*) Infected rabbit brain, at laboratory temperature, keeps its activity in liquids of pH 4 6 to 8.4 (Nicolau and Kopciowska, 1930 *c*)

Ultraviolet irradiation Virus, in a fluorescent medium, is very sensitive (Levaditi, 1942 *b*) Atomized virus is sensitive to UVL of wave length 2537 Å (Edward, Lush, and Bourdillon, 1943)

Reaction to Chemical and Biological Agents

Glycerol Strains vary somewhat in their resistance to 50 per cent glycerol, while some become modified in virulence, others retain their activity almost unimpaired for a year or more Levaditi and Harvier (1920 *b*) showed that the brain

OTHER PROPERTIES OF HERPES VIRUS

Filtrability

The virus passes the following filters· Nordmayer-Berkefeld (Luger and Lauda, 1921 *d*), Chamberland L_1 (Levaditi and Harvier, 1920 *a*, Blanc, 1921), and L_2 (Perdrau, 1925), Mandler (Greenbaum and Harkins, 1925) Ward and Tang (1929) have shown it to be more readily filtrable when suspended in broth than in saline, and using this technique, found Berkefeld V filtrates to be infective. The virus was shown to pass through collodion sacs by Levaditi and Nicolau (1923 *b*), and through collodion membranes by Zinsser and Tang (1927) before the introduction of Elford's gradocol filters.

Tissue Cultivation

The virus grows readily in media of the Maitland type (Gildemeister *et al*, 1929, Andrewes, 1930; Haagen, 1931, 1933; Saddington, 1932). The maximum growth occurs at 34°–35° C. (Thompson and Coates, 1942). Virus can be propagated on agar slopes with finely minced chick embryo tissue on the surface (Cheever, 1939, Cheever and Willmert, 1942).

Growth in the Fertile Egg

Herpes virus grows on the chorio-allantois and produces pocks, typical intranuclear inclusions being found histologically (Saddington, 1932, Dawson, 1933, Burnet, 1936, Burnet and Lush, 1939 *a;* Burnet, Lush, and Jackson, 1939 *a*, *b;* Anderson, 1940, Smith, Lennette, and Reames, 1941).

The HF strain is said to produce larger foci than most freshly isolated strains, the foci are proliferative-necrotic, and, microscopically, inflammatory cells can be found in the mesoderm (Beveridge and Burnet, 1946)

Burnet and his colleagues have used pock counting as a method of titrating virus or virus-antiserum mixtures (see also Schaffer and Enders, 1939, Gallardo, 1943, Florman and Trader, 1947, Rose and Molloy, 1947).

Schaffer and Enders (1939) have reported investigations on the effect of antiserum on chorio-allantoic infection. They found that serum inoculated on the membrane five minutes before, or at the same time as virus, prevented infection When serum was added five minutes later, 4–8 per cent of the virus was *already* "fixed" by the tissues, and by 18 hours all the particles were insusceptible to the neutralizing action of the serum.

After adaptation to chorio-allantoic passage, virus generalizes and brings about *death of the embryo in 3–5 days* *Anderson (1939, 1940) in one series* of passages, found that when the neurotropic HF strain had been passed serially 70 times, its virulence for the embryo was greatly increased, while keratitis-producing power *for the rabbit was lost* *Beveridge and Burnet (1946)* working with a mouse passage strain of HF, found that virulence for mice was lost, although it still produced keratitis in the rabbit

After yolk sac inoculation in 8-day eggs, the virus generalizes and can be recovered in high titer from the amniotic and allantoic fluids (Nagler, 1946, Rose and Molloy, 1947) After 3 days incubation at 35° C, the amniotic fluid contained at least 10^7 living particles per c c The protein content of the fluid in living infected 11-day embryos was 0·012 per cent compared with the normal figure of 0·005 per cent

The effect of amniotic inoculation has been investigated by Burnet (1940), and Anderson (1940) *The latter also infected embryos subcutaneously, by scarification,* and intracerebrally Beveridge and Burnet (1946) found that embryos inoculated when 8 days old died in 48 hours, and the amniotic fluid titered 10^{-7}–10^{-8} per c.c , 6-day embryos also died and gave fluid of high titer

the virus neutralization technique or by complement fixation (see, e g, Zinsser and Tang, 1929, Hudson, Cook, and Adair, 1936; Gildemeister and Ahlfeld, 1937, Burnet and Williams, 1939, Armstrong, 1943, Humphrey and McClelland, 1944)

Virus neutralizing antibody can be detected by inoculation of the chorio-allantois with serum-virus mixtures (Burnet and Lush, 1939 *b*, Burnet and Williams, 1939, Gallardo, 1943; Nagler, 1946, Rose and Molloy, 1947). Alternatively, the mixtures can be inoculated intracerebrally in mice (Smith, Lennette, and Reames, 1941, Scott, Steigman, and Convey, 1941, Evans, Bolin, and Steves, 1945, Slavin and Gavett, 1946 *a*). Rabbits or other susceptible animals can also be inoculated intracerebrally or by other routes.

Antibodies are less commonly found in the sera of children than of adults, antibody develops in the sera of children during convalescence from primary herpetic stomatitis (Weyer, 1932–3; Burnet *et al*, 1939; Burnet and Williams, 1939, Gallardo, 1943, Buddingh, 1946, Nagler, 1946, Rose and Molloy, 1947). Antibody is very much more frequent in children from crowded areas Once developed, herpetic antibody probably remains throughout life, so that approximately 75 per cent. of sera from any unselected group of adults show virus neutralizing properties (Andrewes and Carmichael, 1930)

Tests on selected subjects show that the serum of herpetic persons regularly contains antibody, while it is absent from the serum of normal people. Brain (1932) demonstrated this by the complement fixation test, and it can be shown also by virus neutralization tests (e g, Andrewes and Carmichael, 1930, Burnet and Lush, 1939 *a*, Burnet and Williams, 1939, Nagler, 1946).

It has been said that antibody is usually absent in the sera of patients suffering from encephalitis lethargica (Gay and Holden, 1931).

When antibody is present in the sera of children or adults it is so to high titer, sera are rarely found where antibody is present to a low or moderate titer only; this is because herpes is a chronic infection and presumably there are constant stimuli to antibody production (Burnet and Lush, 1939 *b*, Burnet, 1945).

Thomas (1941) has reported the apparent cure of a case of recurrent herpes by intracutaneous desensitization with autogenous blood serum withdrawn at the height of an attack.

Skin test. Nagler (1944, 1946) has shown that herpetic subjects give a positive skin reaction to a heated antigen prepared from amniotic fluid This positive reac-

Herpetic Immunity in Animals

In marked contrast to the state of affairs in man a general immunity can be readily developed in certain animals This immunity arises either on recovery from infection or after protective vaccination. The exact rôle which serum antibodies and tissues play in the development of this immunity is uncertain There is no evidence that leukocytes are concerned (Silber and Shubladze, 1946).

A. Natural immunity.

Rabbits are generally regarded as being universally susceptible to herpetic infection, but a case has been recorded in which injection by numerous routes was withstood, there having been no previous contact with the virus in any way, while the serum of the animal exhibited no virus neutralizing properties (Teissier, Gastinel, and Reilly, 1928) The newborn of actively immunized mice are immune to skin infection for 2 weeks after birth (Andervont, 1929 *b*) They also resist nasal instillation, resistance is probably due to the imbibition of antibody in the milk (Berry and Slavin, 1943).

of a rabbit suff [illegible] preservation in glycero [illegible] bstance in fresh brain wh [illegible] troyed by glycerol, thus "unmasking" the virus. Da Fano and Perdrau (1927) found that the brains of rabbits suffering from chronic herpetic encephalitis might prove to be inactive when fresh, but active after glycerolization, Perdrau (1938) found that *alumina* would also activate virus. Similar results have been recorded by Nicolau and Kopciowska (1930 *b*).

Electrolytes. Virulence is rapidly lost in salt solutions at 37° C., complete inactivation often taking place in 4 to 5 hours. Serum (Zinsser and Tang, 1926) and distilled water (Gay and Holden, 1929) do not, however, have this property.

Oxygen. Rapid deterioration of virus occurs in the presence of oxygen, but under anaerobic conditions viability is prolonged (Zinsser and Tang, 1929), especially if cysteine is present (Zinsser and Seastone, 1930). Suspensions in broth can be inactivated by oxidation and reactivated on subsequent reduction (Perdrau, 1932).

Other agents. The virus is adsorbed and neutralized by carmine powder, adsorbed but not neutralized by charcoal, and is precipitated with the carbon dioxide globulins (Gay and Holden, 1929). Bile destroys the virus, but it is resistant to neutral red (Blanc, Tsiminakis, and Caminopetros, 1921). Herpes virus is sensitive to the virus inactivating agent of nasal secretion, saponin, sodium desoxycholate, and sodium lauryl sulphate (Burnet, Lush, and Jackson, 1939 *b*; Burnet and Lush, 1940) It is inactivated rapidly by potassium salicylate (Cooke and Best, 1941), and ascorbic acid (Holden and Resnick, 1936, Holden and Molloy, 1937).

Chemotherapy Prontosil and similar compounds had no prophylactic or curative effects in rabbits injected intracranially with virus (McKinley *et al.*, 1939). Vitamin C, pitressin, and sulfanilamide were ineffective in the treatment of encephalitis (Flexner, Chassin, and Wright, 1940). Cutting *et al* (1947) found antibiotics and a number of chemotherapeutic drugs to be ineffective in eggs and mice.

Affinity for Epithelial Tumors

Herpes survives locally when inoculated into an epithelioma of the mouse, thus resembling neurovaccine (Levaditi and Nicolau, 1922 *b*). In contrast to neurovaccine, *however, herpes is not found locally in Shope's papillomatous tumor* whether injected into the tumor or intravenously (Levaditi and Schoen, 1936)

IMMUNITY

Herpetic Immunity in Man

As recurrent attacks are so common one would not expect any significant immunity to be conferred by herpetic infection It has, however, been shown that recurrent vesicles are not found in precisely the same spot on each occasion (Dubreuilh, 1900) This probably does not indicate local immunity, as two separate injections of virus have been given at a month's interval into the same site, with positive results on each occasion (Teissier, Gastinel, and Reilly, 1926 *a*).

Prevention and treatment Brain (1937) has suggested that when the antibody content of the serum of persons subject to herpes falls below a certain level active herpetic lesions occur To prevent such attacks, therefore, the antibody content should be raised by vaccination, either with the patient's own vesicular fluid (Hruszek, 1933), or with a 10 per cent formolized suspension of infected guinea-pig pads (Brain, 1936), or other tissue containing active virus (Teissier, Gastinel, and Reilly, 1926 *a*). It seems that some benefit may follow such treatment in cases of recurrent herpes. Repeated vaccination with calf lymph is said to cure recurrent herpes (see Foster and Abshier, 1937, Woodburne, 1941).

Serum antibodies Antibodies can be detected in the sera of convalescents by

1929 *b*). Kanazawa (1938) has succeeded in immunizing them with phenolized virus injected intraperitoneally, intracerebrally, or cutaneously. Burnet and Lush (1939 *c*) immunized mice by intranasal instillation of living, and intraperitoneal inoculation of formol-killed, virus.

C. Active immunity to superinfection (Magrassi's phenomenon).

A considerable amount of work has been carried out on the reaction of the rabbit to 2 separate injections of herpes virus at definite intervals (Magrassi, 1935, 1935–6 *a*, *b*, Doerr and Seidenberg, 1936–7; Hallauer, 1937, Doerr and Kon, 1937) Thus, when a second (intracerebral) injection is given a few days after a preliminary (corneal or intradermal) injection, mutual extinction occurs and no characteristic encephalitis develops The original papers are extremely detailed but the following are their main findings

1. *Corneal inoculation.* After a corneal inoculation with a nonencephalitogenic [illegible]

its normal pathogenic action is withstood, the animal remaining well This rapidly developing immunity is "solid" and permanent (Magrassi)

If instead of a nonencephalitogenic an encephalitogenic strain be used for the preliminary corneal inoculation, then the second intracerebral injection not only fails to produce encephalitis itself, but prevents its development as the natural sequel of the primary keratitis Doerr and Seidenberg showed that this immunity to cerebral reinjection arises earlier when an encephalitogenic strain is used for the primary corneal inoculation than one which is nonencephalitogenic. They showed that this is due to the fact that of the two strains the more virulent spreads to the brain more rapidly Spread occurs along nerves, and virus can be demonstrated in such nerves for 2 to 4 days, "autosterilization" occurs thereafter and the nerve (like the brain later on) appears devoid of virus

Examination (by animal injection) of brains soon after the second (intracranial) injection shows that the virus is actually demonstrable for some days Immunity to superinfection only arises when virus is actually present in the brain at the time of the second injection Eventually "auto-sterilization" occurs and the brain appears to be virus-free. Actually, however, the reaction is more a partial neutralization than a complete destruction of virus, because the brain is still capable of inciting the production of active immunity on injection into other animals.

It is not possible to prevent the development of consecutive encephalitis after corneal inoculation if the second inoculation is given on the other cornea Mutual extinction does not occur in this case even though virus from each injection must meet in the brain

2 *Intradermal injection* Following a single intradermal injection of virus, either encephalitic or myelitic symptoms develop on the eighth to tenth day. It is possible to prevent the occurrence of these by a second injection of active or formolized virus, but only if given intracerebrally and on the seventh to eighth day This is another example of the 2 infections cancelling each other, and again the mechanism appears to depend on virus neutralization rather than destruction

3 *Possible explanations* The undoubted neutralization which occurs does not appear to be due to any demonstrable virus neutralizing power of the immune brain There is, indeed, some increase of serum antibodies in these animals, but such increase does not seem sufficient to account for the whole phenomenon, because, although formolized virus injected intradermally gives a higher anti-body titer than active virus, animals immunized therewith possess a lower grade cerebral immunity than do those receiving living virus It should be noted that the inocu-

B. Active immunity following infection and vaccination.

On recovery from herpetic infection rabbits are usually immune to reinoculation. Thus, after an attack of keratitis in the rabbit the same eye is immune to further herpetic infection (Lowenstein, 1919, 1920, Blanc and Caminopetros, 1921 *a*). It seems probable that if the initial keratitis is sufficiently severe the other eye also will become immune (Doerr and Vochting, 1920); and even subdural injections may be without effect (Blanc and Caminopetros, 1921 *b*, Doerr and Schnabel, *1921 a*).

In certain cases, if a second corneal inoculation is performed on an eye recovered from keratitis, an intense keratoconjunctivitis develops in a few hours, this being an example of hypersensitivity (Fontana, 1921).

Numerous authors have succeeded in immunizing rabbits against reinfection (Perdrau, 1925, 1938, Zinsser and Tang, 1926, Fournier and Levaditi, 1928, Teissier *et al.*, 1928, Urbain and Schaefer, 1929, Vieuchange, 1936, Hallauer, 1937, Magrassi and others as described below)

The inoculum usually consists of living virus, either fresh or glycerolated, formolized virus may be of use (Urbain and Schaefer, 1929). To produce immunity repeated large doses are usually given, but Perdrau (1925) reported that immunity developed in *11 days after one intradermal injection and lasted over* 3 months

As *regards the routes of injection, a great variety is available: intradermal,* dermal, intranasal, intravenous, corneal, instillation of the external auditory meatus, intramuscular, intraneural, and subcutaneous injections may all be used.

If the resultant immunity is marked, even intracranial injection is resisted, but if it is less well developed, intradermal injection only may be tolerated

Perdrau (1925) found that immunity (tested by intracranial injection) was most readily acquired when virus was injected intradermally. As regards the actual source of the virus used for immunization by this route, human vesicle fluid proved inactive, but infected rabbit brain was active. After one passage through rabbit brain, however, the human vesicle fluid was able to induce immunity. If a second intradermal injection was given within 11 days of the immunizing dose, encephalitis developed

Following intracranial injection of immune rabbits the virus cannot be recovered from the brain after 2 hours During the first few hours following such an injection an increasing polymorph infiltration is found, but this soon disappears as the virus is overcome (Nicolau and Kopciowska, 1929).

It has been suggested that the mechanism of immunity following vaccination depends on a mild subclinical infection, as rabbits frequently die of encephalitis during the course of injections (Perdrau, 1925, Fournier and Levaditi, 1928) Animals may even die of encephalitis 6 months after injection with living virus, although earlier they have been proved resistant to an intracerebral injection, active free herpetic virus can be isolated from the brains (Perdrau, 1938) Hallauer (1937) showed that immunity of the spinal cord advanced *pari passu* with the spread of active virus therein Animals injected with neutral mixtures of virus plus immune serum are not rendered resistant (Zinsser and Tang, 1926).

Guinea-pigs can be readily protected against subsequent infection by inoculation of virus on the plantar skin This immune state develops after about 5 days and lasts *for at least 130 days (Bedson and Crawford, 1927).* These animals can also be immunized by the intraperitoneal route (Olitsky and Long, 1928), or by scarification on the hairy skin followed by intramuscular injection (Bedson and Bland, 1928). Bedson (1931) found that formol-killed virus was effective.

Monkeys, too, can be rendered immune after repeated intradermal injections, the immunity lasting at least 4 months (Teissier, Gastinel, and Reilly, 1925)

Mice have been immunized by repeated intraperitoneal injection (Andervont,

injected prophylactically prior to corneal inoculation (Kitchevatz, 1934) It may also prevent herpetic infection in mice (Andervont, 1929 *b*, Gildemeister and Ahlfeld, 1937, Burnet and Lush, 1939 *c*, Berry and Slavin, 1943). Guinea-pigs may be hyperimmunized, and their serum can confer passive immunity (Bedson and Crawford, 1927). Sera can be produced in chickens (Anderson, 1940).

Evans, Slavin, and Berry (1946) have studied passive immunity in 2-week-old mice infected in the foot pad. When serum was given intraperitoneally at the same time as the virus was inoculated, most mice were protected, the virus being presumably destroyed locally. Serum given at 12 hours after virus inoculation had some but less effect, while up to 48 hours, the infection was retarded. The partly protective effect of serum given after about 24 hours was probably due to a direct action on virus in the CNS, this was confirmed in experiments where serum was given to mice in whom the primary site of inoculation was amputated, and where a local action was thus excluded. They suggest that serum can protect young mice by neutralizing virus before it enters epithelial cells, by preventing virus in epithelium from invading nerves or by a direct antiviral effect in the cells of the CNS.

F. Properties of tissues of immune animals.

It has been claimed that the brains of actively immunized rabbits can neutralize virus *in vitro* (Levaditi and Nicolau, 1922 *a*, Zinsser and Tang, 1926, Teissier *et al*, 1928, Urbain and Schaefer, 1929), and that the adrenal of such animals has the same property (Teissier *et al.*, 1928)

usual incubation period was markedly shortened This effect he attributed to an "aggressin" It appears that this "aggressin" does not exert its full action in the fresh state, but only after glycerolization.

RELATIONSHIP OF HERPES FEBRILIS TO OTHER VIRUSES

1. Zoster

On clinical grounds there appears to be little similarity between herpes febrilis and zoster, although both are characterized by vesicle formation Thus, the course of zoster is more prolonged than that of febrile herpes, zoster most frequently occurs on the trunk, while herpes does so rarely, the bullae of zoster are considerably larger than the small vesicles of herpes, zoster occurs unilaterally, along the distribution of a nerve, and pain is a marked feature, while herpes usually occurs at the mucocutaneous junctions and may be relatively painless, zoster seldom recurs, but herpes febrilis does so frequently.

Herpes virus gives rise to characteristic lesions in animals which cannot be produced with zoster virus It should be remembered, however, that both diseases are thought to spread via the nerves, and changes in the gasserian ganglion have been recorded in cases of herpes febrilis (Howard, 1905), as well as in zoster In addition, Teague and Goodpasture's (1923–4) work showed that the eruption produced by herpes febrilis could be made to assume a zoster-like distribution (*vide supra*)

Immunological observations do
lationship exists between the two vi
zoster fluid induces no immunity to
and Lauda, 1921 *b*), and no comp
and herpetic sera (Netter and Urbain, 1924)

There seems to have been little support for the suggestion of Teague and Goodpasture that cases of an intermediate nature occur which cannot definitely be allotted to either group, and that zoster is produced by a virus differing from that

lations can be brought to mutual extinction only if the second is given intracerebrally, no other route being effective.

The precise explanation of this phenomenon of immunity to superinfection may depend on a cerebral tissue reaction of a type hitherto unrecognized It appears to fall into the category of the "interference phenomenon," a number of other examples of which are described in this book.

D. Serum antibodies.

There is definite evidence that herpes virus can unite with antibody *in vitro* (Bedson, 1928, Smith, 1930). Thus, the latter showed that herpes-infected tissue absorbs the *homologous antibody from a specific serum, and from a mixture of* antiserum to herpes and vaccinia, leaving the antibody to vaccinia. Burnet and Lush (1939 *a*) have carried out studies on the union between virus and herpetic antibody—making use of the egg membrane as an indicator of the presence of virus They found that virus is inactivated by antiserum, and if enough time is allowed, an equilibrium is reached where concentration of antiserum × percentage of survivors is constant. The union was found to be reversible by dilution for some time after the equilibrium was attained, but with strong mixtures partial irreversibility subsequently developed.

Complement fixation occurs between herpes hyperimmune serum (guinea-pig) and virus antigen, when at least 4 MHD of complement may be deviated (Bedson and Bland, 1929 *b*). The fixation time should be prolonged, overnight in the refrigerator being recommended Stroian (1929) found that serum and extracts of testicle, ovary, adrenals, and other organs of actively immune rabbits fixed complement in the presence of herpetic antigens. Complement fixation has also been demonstrated with the serum of an actively immunized monkey, the antigen being prepared from herpetic crusts (Todorovitch, 1925).

Viruciding have been demonstrated in the serum of actively immunized rabbits by a number of workers (Flexner and Amoss, 1925 *c;* Zinsser and Tang, 1926; Bedson and Crawford, 1927, Schultz and Hoyt, 1928, Gay and Holden, 1929. Smith, 1931 *a*). Similar antibodies have been demonstrated in *actively immunized* guinea-pigs (Bedson and Crawford, 1927, Bedson and Bland, 1928), and mice (Gildemeister and Ahlfeld, 1937). Burnet and Lush (1939 *c*) titrated neutralizing antibody by the chorio-allantoic technique. Similar results are obtained by titrating antisera on the chorio-allantois or by inoculating mixtures of serum and virus intracerebrally in mice (Schaffer and Enders, 1939).

Opsonic experiments have also been carried out (Jamuni and Holden, 1934) These workers found that normal leukocytes aided in the inactivation of virus in the presence of immune serum (rabbit) Analogous results were obtained, however, *with immune cells and normal serum Either immune or normal cells* possessed a greater virucidal effect than immune serum alone, and this they attributed to opsonization of the virus particles

There is at present some difference of opinion concerning the rôle of antibodies in the mechanism of herpetic immunity Certain work, mentioned above, supports the view that immunity is dependent on the presence of herpes virus in the immune tissues (infection immunity) Other work has, however, tended more to support the rôle of antibodies Burnet and Lush (1939 *c*) for example, working with mice, concluded that the production of antibodies was the main factor in conferring acquired immunity—because immune bodies were produced by immunization with dead virus, and *passive immunity could be conferred* by high-titer herpetic antiserum

E. Hyperimmunization and passive immunity.

Rabbits may be hyperimmunized by repeated injections of virus (Zinsser and Tang, 1926) Such serum may have a favorable effect in preventing keratitis, if

strains have been obtained (e g., by Flexner and Amoss, 1925 *b*) which are as encephalitogenic as any isolated from encephalitis Florman and Trader (1947) found that two strains isolated from nervous disease in man were not more neurotropic in test animals than a mouth strain. The mouth strain was as dermotropic in the guinea-pig as was a nervous strain They concluded that the neurotropic and dermotropic properties of the virus depend on the host and not on the intrinsic nature of the strain. Further, although after corneal inoculation with herpes strains no symptoms of encephalitis may appear, the virus can none the less be found in the brain (Magrassi and others, *vide supra*)

Strains of virus have been isolated from encephalitis only on relatively few
...on with herpes possible,
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tity of the viruses isolated from encephalitis with those of known herpetic origin. This identity is now almost universally admitted as they cannot be distinguished by any known test.

Herpes virus can cause meningitis and encephalitis in man, but this syndrome does not resemble encephalitis lethargica in any essential respect.

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of herpes only in virulence It is *probably safe to conclude, therefore,* that although affection of nerves occurs in both diseases, the viruses of herpes febrilis and zoster are distinct.

2. Varicella

Antigens made from herpetic vesicles do not fix complement with the convalescent sera of chickenpox cases (Todorovitch, 1925), vice versa, antigens made from chickenpox crusts give negative reactions with herpetic sera (Netter and Urbain, 1924).

3. Variola-vaccinia

Jenner (see Roxburgh, 1927) noticed that vaccination with calf lymph might fail to "take" in a subject suffering from herpes. This observation receives some support in that herpes is rarely found in cases of smallpox (see Roxburgh). The cornea of a rabbit immune to herpes has been shown to possess a partial immunity to variola; further, the serum of such an immune animal has a virus neutralizing action on variola (Gildemeister and Herzberg, 1925). These observations do not, however, afford adequate *grounds for postulating a relationship* between herpes and variola-vaccinia (for further work, see p 331). Vaccinia and herpes can be passed together cerebrally in rabbits, glycerol treatment of the brain destroys vaccinia long before herpes, so that the two agents can be separated by this means (Levaditi, 1941 *c*).

4. Virus "B"

This question is discussed at length in Chapter CI, here it may be mentioned that Sabin recorded a fatal case of ascending myelitis following a monkey bite (see Sabin, 1934). A virus ("B") was isolated, which although resembling, did not appear to be identical with, that of herpes Haber (1935) thought, however, that virus "B" was probably a genuine herpes febrilis strain.

5. Other Viruses

Herpes is not related to the viruses of infectious feline agranulocytosis (Lawrence *et al.*, 1943), or epidemic keratoconjunctivitis (Sanders and Alexander, 1943).

THE "HERPETICO-ENCEPHALITIC" GROUP OF VIRUSES

The herpes virus is one with both dermotropic and neurotropic properties The dermotropic properties are indicated by the ease with which the cornea and skin may be infected, while its neurotropism is shown by the encephalitis resulting from intracranial or other injections The variability of the characters of herpetic strains has been repeatedly stressed (Levaditi and Nicolau, 1922 *c*, Levaditi, 1922, 1926, Teague and Goodpasture, 1923–4, Flexner and Amoss, 1925 *a*, *b*, *c*, Lépine and Schoen, 1931). While some have only dermotropic, others have both dermotropic and neurotropic tendencies The French authors, in particular, hold that the two affinities are quite dissociable.

This problem is intimately associated with that of the possible relationship between the virus of herpes febrilis and strains isolated from epidemic encephalitis (this is fully discussed in Ch XCII) Here it may be mentioned that Levaditi and his collaborators have concluded that the two viruses form a group designated by them "herpetico-encephalitic" (e g, Levaditi and Nicolau, 1924, Levaditi, Nicolau, and Poincloux, 1924, Danila and Stroe, 1923 *a*, *b*, *c*, *d*, Levaditi, 1926) They suggest that at one extreme are found neurotropic strains which, granted a suitable host, may cause encephalitis, while at the other end of the scale are dermotropic strains possessed of no neurotropic properties Intermediate strains are, of course, encountered

It is questionable, however, whether this distinction really holds good, for

strains have been obtained (e.g, by Flexner and Amoss, 1925 *b*) which are as encephalitogenic as any isolated from encephalitis Florman and Trader (1947) found that two strains isolated from nervous disease in man were not more neurotropic in test animals than a mouth strain. The mouth strain was as dermotropic in the guinea-pig as was a nervous strain. They concluded that the neurotropic and dermotropic properties of the virus depend on the host and not on the intrinsic nature of the strain Further, although after corneal inoculation with herpes strains no symptoms of encephalitis may appear, the virus can none the less be found in the brain (Magrassi and others, *vide supra*).

Strains of virus have been isolated from encephalitis only on relatively few [illegible] contamination with herpes possible, [illegible] be in the fluids of the body. Gen- [illegible] cerned itself with proving the identity of the viruses isolated from encephalitis with those of known herpetic origin. This identity is now almost universally admitted as they cannot be distinguished by any known test.

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CHAPTER XVIII

STOMATITIS AND DIARRHEA OF INFANTS

THIS CONDITION, probably due to a virus related to herpes febrilis, has been described by American workers (Buddingh and Dodd, 1944, Buddingh, 1946, Dodd, 1946). The following account is largely taken from that of Buddingh (1946).

Clinical Features

The highest incidence of attack is in those under 6 months of age. Children over 7 months usually show only stomatitis The onset is sudden, and vesicles develop on the tip and under surface of the tongue, and occasionally on the gums and inner surfaces of the lips The stomatitis persists for 7–14 days, with a tendency to show short periods of remission Young infants are more likely to exhibit diarrhea as the main symptom, the stomatitis being often overlooked The loose stools continue for 4–10 days.

Epidemics may occur in nurseries These epidemics are usually mild, but may be severe, especially in premature babies. Dodd (1946) described an outbreak with a death rate of 50 per cent. Adults in close association with the disease may be affected with stomatitis.

The virus has been recovered from the vagina of mothers of infants who develop diarrhea shortly after birth. The virus has been recovered also from urethral discharge, conjunctivae, and the mouth in a case of Reiter's disease

Pathological Features

Gross changes are found primarily in the small intestine. The mucosa is swollen and shows numerous petechiae. There is ulceration and some cellular infiltration The lesions of the tongue show focal necrosis, and an inflammatory reaction.

Epidemiology

Adults suffering from stomatitis, or without clinical evidence of infection, who harbor the virus, may be the origin of nursery epidemics. The virus can also be acquired during birth from the maternal passages It appears possible that the virus is spread in adults venereally.

Properties of the Causal Agent

The causal agent can be found in swabbings from mouth lesions or in stools, or in the vagina of mothers

Animal experiments Rabbits inoculated on the cornea develop a cloudy opacity in 24 hours The conjunctiva is swollen also, iritis may occur The condition clears up in 2–3 days

Strains of the agent have been maintained by serial passage of ground corneae at 48-hour intervals in young rabbits Histologically there is necrosis and an acute inflammation No inclusions can be seen

Diarrhea can occasionally be produced by feeding infective material to young rabbits The mucosa of the jejunum and ileum shows numerous scattered petechiae.

Local reactions can be produced by scarification or intracutaneous inoculation of the pads of guinea-pigs' feet The lesions show necrosis and infiltration with polymorphs

Filtration The agent is filtrable through Berkefeld V candles.

Eggs. It cannot be transmitted to fertile eggs

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CHAPTER XIX

FOOT-AND-MOUTH DISEASE IN MAN[1]

NUMEROUS CASES of foot-and-mouth disease have been described in man (Siegel, 1891, Bertarelli, 1908, Pape, 1921; Fahr, 1923, Trautwein, 1929–30, Fessler, 1934, Curschmann, 1938, Kanyó and Oláh, 1938, Richter, 1938, Rochaix and Delbos, 1938 *a*, *b*, *c*, and *vide infra*). In addition, various authors have published general reviews, often with bibliographies (McBride, 1869, Bussenius and Siegel, 1897, Gins and Krause, 1923–4, Arkwright, 1928, Sée, 1934)

It is probable that many of the recorded cases were not true infections due to the virus of foot-and-mouth disease However, there is a number of cases recorded where the virus has been isolated from the patient, or where the convalescent serum has shown a rise in specific antibody (Flaum, 1939, Bojlén, 1941).

Clinical Features

Incubation period The incubation period is usually from 2 to 5 days, but may be longer.

Onset The patient is usually quite ill, with headache and pyrexia The mucous membrane of the mouth feels dry and hot, and hypersalivation occurs. The skin may be itchy

Course. After a day or so these invasive symptoms subside and the characteristic vesicles appear in the mouth, and sometimes elsewhere (*vide infra*) The lesions a 2 to 3 days t with under- r ! om lasts for more than 8–15 days

Site of the lesions.

Buccopharyngeal cavity The characteristic vesicles are freely distributed on the mucosa of the lips and gums, tongue, and pharyngeal walls, and may attain considerable size before ulceration occurs The tongue and lips are often considerably swollen and painful The effect of these lesions is to provoke salivation, which may be so intense as to cause serious discomfort to the patient. The pharynx is often acutely inflamed and ulcerated, rendering swallowing painful and difficult The breath is usually offensive.

Limbs Vesicles occur in the greatest numbers on the tips of the fingers on the volar aspect, and at the base of the nails (Clough). Another characteristic site is between the fingers, and the lesions may also occur between the toes In severer cases the forearms and legs may also be affected

Other sites A number of authors have noted the presence of lesions on the conjunctivae The eyes have usually been very watery, and the lids gummed together

Histology of the Vesicle

The histology of the vesicle has been well described by Sutton and O'Donnell (1916), and the changes are as follows The cornified layer is parakeratotic, while

[1] Foot-and-mouth disease is predominantly an animal condition Consequently, we shall restrict our description to cases of human infection The properties of the virus are only briefly discussed

Immunity

In human cases, specific neutralizing antibodies develop in convalescence.

On recovery from keratitis, the rabbit's cornea becomes resistant to reinfection in about 3 weeks.

Specific neutralizing antibodies develop in the serum of rabbits immunized by intravenous injection.

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the stratum lucidum is thickened and spongy. The cells of the rete malpighii are swollen and increased in numbers, many containing fluid material The vesicle is developed in the deeper layers of the rete; it is somewhat irregular in outline, and is subdivided into loculi by septa. The basal cell layer is almost obliterated, while the prickle cells are infiltrated with fibrin and leukocytes.

Epidemiology

Geographical distribution. Human cases of foot-and-mouth disease have been reported most frequently from Germany. Thus, Bussenius and Siegel (1897) referred to 600 supposed cases in man between the years 1887 and 1894. It is doubtful, however, whether the condition is so common at the present time, although there have been many further descriptions of cases Cases have been reported from the following countries e g., the United States (Clough, 1915, Sutton and O'Donnell, 1916, Sutton and Sutton, 1935), Hungary (Mollow and Pentschew, 1930, v Scheitz, 1934); France (Cadiot, 1913; Sée, 1934; Cambessédès, 1935), Switzerland (Roch, 1939); Scandinavia (Flaum, 1939; Kling, Huss, and Olin, 1939; Bojlén, 1941), Poland (Dlugosz, 1943) In England a number of cases have been described (McBride, 1869, Gibbons, 1924; Dlugosz, 1943). A case has been recorded from the Belgian Congo (Gillain, 1936).

Spread Man can become infected with the virus in various ways. The infection can be spread directly, through contact of the abraded skin with infected animals or their saliva or urine. In these cases the virus usually enters through the skin of the hands, most readily through cuts and abrasions The virus is not of high infectivity for man, and cases in experimental stations are very unusual, despite the risk of contamination of the hands.

The virus may also enter the body by the digestive tract. The usual source of infection in these cases is milk, butter, cheese, or other milk products coming from diseased animals

Fomites may also play a part in the spread of infection

Isolation of the Virus from Human Cases

The diagnosis can be made quite simply by withdrawing vesicle fluid in a capillary tube, and inoculating experimental animals, especially guinea-pigs on the pad (Pancera, 1922, Gerlach, 1924, Trautwein, 1929–30, Fessler, 1934; v Scheitz, 1934, Richter, 1938). It is said that better results are obtained if the ground-up vesicle wall can be used for inoculation, instead of fluid (Bojlén, 1941) On recovery from infection the animals can be "challenged" with known antigenic types, so as to ascertain the infecting type Cattle may also be infected with human material (Bertarelli, 1908, Pancera, 1922)

Serology

The serum of convalescents fixes complement in the presence of a suitable antigen, e g., vesicle fluid, or infected animal tissue (Kanyó and Oláh, 1938) A virus neutralization test can also be performed Serum for these tests should be taken 10–20 days after onset

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[illegible] et of pyrexia, [illegible] the conjunc- [illegible] The lacrimal caruncle is usually swollen and shows small Koplik-like spots (*vide infra*), which may also occur on the plica semilunaris (Herrman). Brownlee has stressed the diagnostic value of swelling of the lower palpebral conjunctiva.

4 *Koplik's spots.* These lesions, described by Koplik of New York in 1896, are one of the best known of the prodromal manifestations. They occur on the buccal mucosa of the lips as bright red spots, with a central bluish-white streak, appearing primarily opposite the first molar teeth. They vary from a half-dozen to sufficient to cover the whole of the buccal mucosa With the onset of the rash the spots be- [illegible]

[illegible] fore the rash 47 36 per cent. of patients showed the spots, 2 days before 73 per cent., and 1 day before, they occurred in 100 per cent of cases The spots may occur in the colon (Hobson, 1940, Corbett, 1945).

5. *Pharyngeal signs* During the early stages of invasion the fauces appear red and the soft palate, uvula, and posterior pharyngeal wall show scattered red-brown spots. Herrman (1914, 1915) has drawn attention to tonsillar spots, which he found in 40 per cent. of cases They occur as bluish-gray to white spots varying in size from a pin point to a pin head

6 *Prodromal rashes.* These rashes have been described by various authors (e g, Adkins, 1899, Rolleston, 1905, Stimson, 1928). They may occur on the first day or so of the fever as fine macules, papules, urticarial lesions, scarlatiniform spots, or blotchy erythemas. They are to be found in 30–50 per cent. of all cases Rolleston (1905) mentions the following characteristics of these rashes

(*a*) They appear early, within the first 2 days of the disease, and frequently before Koplik's spots, thus having great diagnostic value.

(*b*) The erythematous and urticarial lesions are transient and may only last for a few hours, although the scarlatiniform, macular, and papular rashes last for a day or more.

(*c*) The distribution is localized, but there is no elective site, and any part of the trunk or limbs may be involved, the face and neck are rarely affected

(*d*) The spots are polymorphic, lesions such as macules and papules may occur together.

(*e*) The rash is not painful or itchy.

7 *Catarrh* The eyes are usually "running" and the conjunctivae suffused. Rhinitis, pharyngitis, bronchitis, and enteritis all commonly occur. Mayerhofer (1932, 1934), in particular, has stressed the diagnostic value of angina in the prodromal period Laryngitis may develop in the preeruptive stage and lead to confusion with diphtheria Box (1933) has described a suffocative catarrh which may develop prodromally, the temperature is raised, the child coughs, is dyspneic and cyanosed, and expectorates profusely, the mortality rate is high. The tympanic membrane may be congested and there may be earache (see Bespaloff, 1937).

Course of the Disease

After 3 to 4 or more days during which prodromal symptoms have been noted, the characteristic rash and other manifestations appear The rash may be delayed in its appearance, and Selby (1936) has recorded cases in which it was noted 9 and 11 days after the onset of prodromal symptoms

The rash.

Many excellent descriptions of the well-known rash are available and the features need only be summarized here (see, e g, Rolleston, 1929) Most frequently

CHAPTER XX

MEASLES (MORBILLI)

THE CONTROL of measles by ordinary public health measures has proved a notable failure, but a powerful prophylactic method has been available for some years By injection of immune measles serum and other biological products it is now possible to prevent cases of measles The fact remains, however, that such measures have not seriously lowered the incidence of the disease, which still remains a major problem to public health administrators. With regard to literature, the work of Thomson and Thomson (1931)[1] is by far the most complete and should be consulted by those desiring further references Other general reviews have been published (McCartney, 1927; Rolleston, 1929, Smith, 1944).

CLINICAL FEATURES

Incubation Period

This is relatively long, about 10 to 12 days, with outside limits of 7 to 21 days (see, e g , Lewy, 1920, Thomson and Thomson, 1931; Stocks, 1931).

The percentage of incubations of 15 days or more was found to be significantly greater in the 12–23 month age group (Stillerman and Thalhimer, 1944).

Illness of infection Goodall (1925) recorded some 7 cases in which children developed pyrexia, sneezing, injection of the conjunctivae, and sometimes a morbilliform rash, within a few hours after contact with a case of measles. After the appropriate incubation period the usual symptoms of measles developed. Erdheim (1926 *b*) made similar observations, noting signs of catarrh several days before the rash Another series of cases was described by Abercrombie (1929) in which some 12 to 13 days before the appearance of the rash children developed fever, gastro-intestinal disturbance, bronchitis, sore throat, and earache Meyer and Greenthal (1930) recorded 4 possible cases of this illness of infection (see also *M.R.C Report*, 1938) The exact explanation of these cases is obscure, but Orel (1925–6) has suggested that the symptoms are due to a mixed infection

Prodromal Symptoms

During the stage of invasion the child is acutely infectious and spreads the disease to numbers of contacts, as isolation is seldom effected until too late The prodromal period may begin at any time up to one week before the development of the rash, although it seldom lasts longer than 3 to 4 days A number of affections occurs in this period which point to the true nature of the illness

1 *Blood* During the early part of the incubation period there is a leukocytosis, which gives place to a prodromal lymphopenia

2. *Pyrexia.* Pyrexia is frequently the first symptom of illness noticed in a child exposed to infection some 10 or more days previously

3. *Eye signs* Changes in the conjunctivae have been stressed by various observers (e g , Koplik, 1896, Herrman, 1914, Brownlee, 1920, Stimson, 1928, 1931) The lower lids appear puffy and there may be a definite congested line across the lower

[1] References are appended at the conclusion of Ch XXII, p 235 et seq

The spleen.

It was suggested by Bleyer (1926) that definite enlargement of the spleen was commonly observed at the time of onset of the rash Friedman (1927, 1931), however, found that this only occurred in 11–12 per cent., was not marked, and appeared independently of the rash.

The sympathetic system.

By studying the oculo-cardiac reflex Lavergne and Debenedetti (1931) noted a sympatheticotonia at the onset of the rash. With the development of the eruption vagotonia supervened, and then there was a return to normal

Varieties of Measles

Morbilli sine morbilli and morbilli bullosi have already been referred to, measles without any catarrhal symptoms (morbilli sine catarrho) may also occur. Rolleston (1929) described the following 3 additional varieties hemorrhagic, ataxodynamic, and suffocative Hemorrhagic measles is very rare and is said to be characterized by bleeding from the lung, bowel, stomach, and other sites (Baker, 1941, Linehan, 1942). Ataxodynamic measles is characterized by hyperpyrexia, delirium, and prostration Suffocative measles presents from the start the picture of a severe dyspnea without any chest signs (see Nobécourt and Lereboullet, 1931; Box, 1933) This type of affection is usually evident about the second day of the rash, the patient is dyspneic and cyanosed, and coughs and expectorates profusely, these cases are usually fatal.

CROSS INFECTION IN MEASLES

Measles virus appears to predispose the respiratory tract to invasion by pathogenic bacteria. When patients are nursed in "open" wards there are abundant opportunities for cross infection of the upper respiratory tract with the subsequent development of bronchitis, bronchopneumonia, and especially otitis.

The main secondary invader is the hemolytic streptococcus, and Allison and Brown (1936) found 51.2 per cent. of patients to be so infected

This question of cross infection in measles wards has been extensively investigated by a group of workers in London fever hospitals. Thus, Wright, Cruickshank, and Gunn (1944) found the predominant organism in ward dust to be a Type 6 hemolytic streptococcus This organism accounted for over 90 per cent. of cross infections, and all the late otitis cases. They showed that the oiling of floors alone did not reduce the incidence of cross infection with the streptococcus When bedclothes and garments were oiled as well, the cross infection rate dropped to 18 6 per cent, as compared with 73 per cent in a control "unoiled" ward In the control ward, 14 3 per cent. of patients developed otitis due to Type 6 streptococcus, whereas in the oiled ward the incidence was only 2 8 per cent. In a later study, Wright (1945) found that 72 per cent of 65 patients at risk acquired infection with Type 6 hemolytic streptococcus, 44 7 per cent. of the cross infections occurred within 1 week, and 34 per cent. in the second week of hospitalization

It now appears that the conditions favoring secondary infection with hemolytic streptococci vary from year to year For example, Begg, Smellie, and Wright (1947), working at a later date, in another London fever hospital, found the incidence of streptococcal cross infection in an "oiled" ward (12–20 per cent.) almost exactly the [illegible]
was very lo[illegible]
cocci differ[illegible]
Type 6 was rarely found, but other A types, and members of Groups C and G were responsible

The question of control of cross infection is discussed below

the rash appears first behind the ears, and within 24 hours spreads to involve the face, head, and neck; the arms, trunk, and legs are soon involved also. The measles papule is very often crescentic, and the individual lesions usually remain discrete, although confluent patches may be found After 3 to 4 days the rash begins to fade, leaving a brownish discoloration and "branny" desquamation. It is well known that the rash may appear most profusely in the neighborhood of various sources of irritation or skin disease, thus Aitken (1933) has shown that the rash may appear markedly on an area of pigmentation due to ultraviolet radiation.

Morbilli sine morbilli This name is given to cases of noneruptive measles; the patient shows catarrh, Koplik's spots, fever, and prodromal rashes but no typical measles eruption (Rolleston, 1904).

Morbilli bullosi. Bullous eruptions may develop shortly after the first appearance of the rash (see Neff, 1920, Burdick, 1922). Thus, Morton (1921) described a case in which after 24 hours bullous eruptions appeared and spread from the chest to the face, trunk, and limbs, he referred to approximately 20 similar cases.

Kelleher (1933) reviewed various reported cases of morbilli bullosi and considered there were good grounds for regarding them merely as acute pemphigus, and not measles at all. In many of the cases, for example, there were no Koplik's spots, further, many patients had already had a genuine attack of measles Ronaldson (1937) formed the opinion that the reported cases fall into 3 groups·

(*a*) True pemphigus with a morbilliform eruption, such cases not being measles at all

(*b*) Genuine cases of measles with an anomalous eruption, i e, true morbilli bullosi (see also James and Miller, 1938)

(*c*) Measles and pemphigus may coexist.

Pyrexia.

Stimson (1928) has given the following description of the various types of temperature chart that may be found in measles

(*a*) *The typhoid type.* Fever is noted in the prodromal period as the first manifestation of illness The temperature rises steadily, so that after 4 to 5 days and with the appearance of the rash it has reached about 104° F

(*b*) *The malarial type* In this type there is fever for the first day or so of the prodromal period, followed by a return to normal for a short space, with a subsequent rise (see also Paton, 1932)

(*c*) *The severe type.* This reaction is frequent in pneumonic cases, when the temperature rises quickly at the start to about 103° to 104° F. and remains raised throughout the prodromal period

(*d*) *The modified type* This type of reaction is seen in those treated prophylactically with serum (sero-attenuation) The temperature gradually rises to 101° to 102° F and slowly falls again

In all types the temperature usually falls by crisis or rapid lysis when the rash is well developed, any subsequent rise probably indicates the onset of a complication

Catarrh.

The prodromal catarrh is usually considerably aggravated with the advent of the rash. The larynx may be involved during the eruptive stage giving hoarseness, cough, and perhaps loss of voice (see Oliver and Turner, 1933) The child is usually "running" at the eyes and "streaming" at the nose, enteritis is common Certain important complications in the ear and respiratory system may develop (*vide infra*).

son (1931) examined 75 cases by lung puncture. In 46 per cent. of cases influenza bacilli were isolated, in 28.4 per cent. *Pneumococcus*, in 8 per cent. *Streptococcus hemolyticus*, in 4 per cent. *Streptococcus viridans*, and in 11 per cent *Staphylococcus* and *Micrococcus catarrhalis*. Ellison found that the influenzal type of case was distinguished by a very severe toxemia and heliotrope cyanosis

The hemolytic streptococcus has figured more prominently in other series Thus, it was the causal agent of the epidemic of postmeasles interstitial bronchopneumonia described in the United States by Cole and MacCallum (1918). Thursfield (1912–13) found the *Streptococcus hemolyticus* in just over 50 per cent of cases, *Pneumococcus*, *Staphylococcus aureus*, and influenza bacilli constituting most of the remainder. Ritossa (1938), in Rome, found the *Pneumococcus* the commonest organism in respiratory complications

Other respiratory complications.

Lobar pneumonia may sometimes occur. Degen (1937) found empyema in 13 per cent. of autopsies and fibrinous pleurisy in 14 per cent.

2. Central Nervous System

Cases of encephalomyelitis during an attack of measles have been reported by numerous authors (e.g., Benn, 1931; Bernuth, 1928–9, Bregman and Poncz, 1929, Bourne, see Winnicott and Gibbs, 1926, Bridgeman, 1937, Crockford, 1932, Draganesco and Milcoveanu, 1936, see *New Zealand Medical Journal*, 1936, Genoese, 1932, Greenfield, 1928–9, Jenkins, 1929, Lancaster, 1938, Miller, 1931, Neale and Appelbaum, 1927, Parrish, 1936, Peterman and Fox, 1933; Price, 1937, Rosenheck and Barowsky, 1937, Signa, 1929, Sulzer, 1930, van Bogaert, Borremans, and Couvreur, 1932; Chinner, 1940, Geiger and Sappington, 1940, Burton and Weir, 1941, Schaffer, 1941, Litvak, Sands, and Gibel, 1943, Meyer, 1943, Reisman and Rosen, 1943). General reviews of the subject have also appeared (Ford, 1928, Greenfield, 1929, van Bogaert, 1933).

Ford reviewed 113 cases and described 12 from his own experience. Since that time there has been no lessening of interest in the subject, and the number of published cases must now number some hundreds The following account is largely based on that of Ford

Clinical features This complication usually develops as the rash begins to fade, although the onset may be earlier, even in the prodromal stage (Arena, 1946). The temperature rises, and the patient becomes drowsy; rigidity and convulsions are common Mild cases recover rapidly, but sometimes severer symptoms may occur· e g, tremors and other signs of neuromuscular incoordination; paraplegia or hemiplegia, purely meningitic symptoms (Tuà, 1923, Reiche, 1927, Lioret, 1930), the cerebellar syndrome (see, e g, Cole, 1938), myelitis (Babonneix and Lévy, 1931, Miller and Ross, 1931, Kousmine, 1934, Rosenheck and Barowsky, 1937, Ward, 1941, Rydeen and Glaser, 1942, Pearlman and Shirreff, 1944), loss of urinary and rectal sphincter control (Miller and Ross, 1931, Elliott and Elliott, 1933, Barnes *et al*, 1937), multiple peripheral neuritis (Urquhart, 1934, Mohammad, 1936, Lenègre and Delair, 1939)

The cell count of the cerebrospinal fluid is usually raised up to about 200 per c mm lymphocytes, while globulin is definitely increased McCausland and McClendon (1934) carried out encephalography, finding dilated lateral ventricles and an increase in the subarachnoid air spaces As regards prognosis, the mortality varies from 10–30 per cent, about 40 per cent recover completely and 40 per cent. recover with sequelae (Ford, 1928, Norman, 1936, Ford and Guild, 1937, Hamilton and Hanna, 1941, Smith and Trapp, 1944) Stitt (1943) described an interesting case where the symptoms were definitely aggravated by smallpox vaccination

Pathology. The pathological features have been described by numerous authors (e g, Barlow and Penrose, 1886, Wohlwill, 1928, Greenfield, 1929, Walthard, 1929,

COMPLICATIONS

1. Respiratory

Laryngitis.

The laryngitis of the eruptive stage may produce ulceration of the arytenoid cartilages and the vocal cords (see, e.g., Box, 1933). In 13 per cent. of measles autopsies Degen (1937) found evidence of laryngeal ulceration, membrane formation, or obstruction. Edema of the glottis or larynx is usually fatal (Homrighouse and McKee, 1939; Mulvaney, 1942).

Bronchopneumonia.

Bronchopneumonia is the most dreaded complication of measles, especially in young children (see Box, 1933). Thus, in the 1927–8 London epidemic 86 per cent. of all measles deaths in the Metropolitan Fever Hospitals were due to pneumonia (Ellison, 1931). In 100 autopsies Degen found evidence of bronchopneumonia in 96. Below the age of 2, from 30 to 50 per cent. of cases of bronchopneumonia die (see, e.g., *Chevalley, Duchon, and Forestier, 1936*). The disease was very prevalent in the United States in soldiers in World War I (see Cole and MacCallum, 1918).

The disease may develop early, or during the eruptive period, other cases develop later, the eruptive fever falling only to rise again with the onset of bronchopneumonic complications.

Pathology. The pathological features of measles bronchopneumonia have been described by various authors (e.g., by Cole and MacCallum, 1918, Denton, 1925, Kuraya, 1929, Ellison, 1931, Smith, 1931, Box, 1933, McCordock and Muckenfuss, 1933, Moore and McCordock, 1934, Degen, 1937, Milles, 1945).

Naked-eye, the lungs are congested and usually show patches of reddish or gray consolidation, bronchopneumonic in distribution. The larynx, trachea, and bronchi are congested and may contain mucopurulent secretion.

Microscopically a variety of changes may occur, in many cases resembling closely the acute bronchopneumonia of influenza (see Ch. LV). Thus in some the picture is that of a pure virus pneumonia (e.g., Moore and McCordock's and Kuraya's cases). The red patches observed naked-eye prove to be necrotic areas where red cells are present in the alveoli and bronchioles. There is only scanty inflammatory reaction.

In other cases the picture is more of a peribronchiolitis with interstitial inflammatory changes. The interstitial tissue is swollen and infiltrated with a serofibrinous exudate. All coats of the bronchioles are inflamed, swollen, and infiltrated with leukocytes, the lining membranes are usually desquamated and the lumina contain mucopurulent exudate. There are areas of peribronchiolar pneumonia where the alveoli are filled with a fibrinopurulent exudate. The infection spreads throughout the parenchyma from these peribronchial nodules.

The changes in the lung are, of course, the most important, but cellular infiltrations and degeneration occur in the mouth, pharynx, larynx, and bronchial passages as well. During the acute stage mediastinal and subcutaneous emphysema may develop. If the child recovers, there is a strong tendency for fibrosis, often with bronchiectasis, to follow.

Giant cells, described as of the nonspecific fused respiratory epithelium type, may be numerous in the bronchi and bronchioles (Stryker, 1940, Corbett, 1945, Milles, 1945, Pinkerton, Smiley, and Anderson, 1945, Archer and Biggs, 1947).

Bacteriology. The bacteriological findings in measles bronchopneumonia are somewhat complex. In certain cases, undoubtedly, the condition is a virus pneumonia in which the virus has shown an unusual tendency to invade the lower respiratory tract. *In other cases the rôle of cultivable organisms* has been established, and these are probably invaders acting secondarily to the virus. Thus, Elli-

schitsch, 1927; Asherson, 1932; Allison and Brown, 1936, Wright, Cruickshank, and Gunn, 1944, Wright, 1945).

Otitis occurring before the 5th hospital day is not regarded as being due to cross infection, but later cases, developing on and after the 6th day are believed to be so explained. Late otitis is about 4 times as frequent as early otitis The great bulk of otitis cases are due to hemolytic streptococci. At one time in London, Type 6 was very prevalent, and about 1 patient in 5 developed otitis Later this type became much rarer, and the incidence of otitis was only 1/376 cases (see above). Wright (1945) reported that late otitis was much less common in April and May than in December–March

4. Appendicitis

In 1936 Hudson and Krakower reviewed some 31 cases of measles with appendicitis and added 9 from their own experience In 15 cases the appendicitis occurred prodromally, in 12 in the eruptive stage, and in 13 in early convalescence Degen (1937) found 3 cases of appendicitis in a series of 100 autopsies. Later cases have been described by Koch (1937).

A number of authors has recorded the presence of multinucleate giant cells in appendices removed in the p [illegible] berg, 1932; Finkeldey, 1932, Ve [illegible] Hudson and Krakower, 1036, [illegible] the same type as those found [illegible] *infra*), measuring about 100 μ and containing 70 to 100 nuclei arranged centrally.

5. Skin Complications

Apart from morbilli bullosi already discussed, certain other skin complications may develop For instance, a cold urticaria was recorded in 3 sisters by Kobacker and Parkhurst (1935; see also Apert and Broca, 1923). Purpuric lesions have also been reported (see, e g, Kelly, 1922, Laurent, 1933)

6. Other Complications

The following is a list of other complications that may occur. stomatitis, noma of the cheek or pudenda, tonsillitis, [illegible] (Binder, 1931), tetany (Ghosal, 19 [illegible] corded a temperature of at least 110° [illegible] rene (Rolleston, 1925–6, Winstead, [illegible] a series of 100 autopsies Degen (1937) found pericardial effusion in 13 per cent of cases and pericarditis in 4 per cent, also peritonitis in 5 per cent.

CLINICAL PATHOLOGY

The urine frequently gives the diazo reaction 2 to 4 days after the appearance of the rash (Malmberg, 1922–3)

The serum may give nonspecific positive reactions in the Wassermann and Meinicke tests (see Pockels, 1931). Mineral phosphorus is said to be diminished at the onset of the rash to 1 8 mg. per cent, but after 2 to 3 days the level returns to normal (Lesné, Zizine, and Briskas, 1934) Plasma amino-acid nitrogen concentration is usually within the normal range (Farr, McCarthy, and Francis, 1942)

There is a mild leukocytosis during the early part of the incubation period, during the prodromal period there is lymphopenia with relative neutrophilia (Stimson, 1928, Lane, 1930, Benjamin and Ward, 1932, Manson, 1935) At the height of the illness there is a general leukopenia, while during convalescence a lymphocytosis may be noted The appearance of the blood changes may be prevented by the use of immune serum (Gunn, 1942)

The red cells are said to be reduced to about 4,000,000 per c.mm. during the

1930, 1937; Zimmerman and Yannet, 1930, Wilson, see Benn, 1931, Ferraro and Scheffer, 1931, 1932; Erb and Morgan, 1933; Marsden, 1934; Babonneix, Lhermitte, and Trelles, 1934; Moore and McCordock, 1934; Degen, 1937, Finley, 1937, Malamud, 1937, 1939, Ziskind and Schattenberg, 1939, and see Greenfield's article for a full bibliography).

(*a*) *Vascular lesions.* Diffuse congestion and petechial hemorrhages are frequently seen by the naked eye in the cerebral white matter, basal ganglia, midbrain, and pons Microscopically, perivascular hemorrhages and edema may be observed, especially in the pons, these changes being found in the majority of cases. Thrombosis and vascular endothelial swelling have also been reported (Ferraro and Scheffer, 1931).

(*b*) *Perivascular cuffing* The commonest infiltrating cell is the lymphocyte, although fat granule cells and plasma cells may also occur Perivascular cuffing is a variable feature, being more prominent in some reported cases (e g, those of Greenfield, Walthard, 1930; Ferraro and Scheffer, 1931) than in others (e g, those of Wohlwill, Moore and McCordock, Marsden).

(*c*) *Demyelination.* Many of these reported cases showed demyelination widespread in the brain and cord (e g, the cases of Greenfield, Wohlwill, Finley, Moore and McCordock, Zimmerman and Yannet, Marsden, Malamud). Wohlwill made a special study, and found that demyelination occurred under the spinal pia, subependymally and perivenously throughout the central nervous system. Perivascular demyelination was most pronounced in the subcortical white matter of the superior and posterior parts of the cerebrum. Greenfield's case showed demyelination to be most pronounced around medium-sized vessels in the deeper parts of the centrum ovale In Moore and McCordock's case the dorsal border of the corpus callosum and the cervical cord were chiefly involved. The demyelinated areas were usually infiltrated with polymorphs and mononuclears, and there was a reactionary perivascular microgliosis Other authors have reported similar appearances

(*d*) *Nerve cells* With regard to the nerve cells, these are usually more or less normal, although slight degeneration may be noted Ferraro and Scheffer (1932) described a case which differed from the usual type of postmeasles encephalomyelitis in that no demyelination was seen, but there was a pronounced involvement of the gray matter There was a diffuse and acute swelling and degeneration of nerve cells in all parts of the cortex and central masses They likened the appearances to those of lead poisoning

Etiology The most constant histological findings are congestion, hemorrhages, and perivascular demyelination, perivascular cuffing is inconstant. The changes are identical in kind, therefore, with those of the commoner postvaccinal encephalitis, they differ slightly in degree, however Thus, Greenfield states that in postvaccinal encephalitis there is a greater amount of perivascular infiltration and more complete demyelination

It is evidently impossible to discuss the etiology of measles encephalomyelitis as an isolated condition, it must be considered together with postvaccinal encephalitis and that of related conditions (see Ch XXXV) Although Gay (1932) isolated the herpes virus in 3 cases, it is generally held that the condition is due to the measles virus This supposition has been confirmed by Shaffer, Rake, and Hodes (1942) who isolated measles virus from the brain of a 7-year-old boy by inoculation in *M. mulatta* We would suggest that this agent is one of many which cause intravascular thromboses in the central nervous system with resultant demyelination

3. Otitis

Measles is responsible for many cases of otitis media, sometimes giving rise to permanent deafness and its usual accompaniments One may expect from 13–20 per cent. of measles cases to be complicated by otitis (Gardiner, 1924, Urbant-

all measles deaths in fever hospitals were due to pneumonia (Ellison, 1931). In 100 autopsies Degen (1937) found 96 cases of bronchopneumonia The main secondary invader in these cases is undoubtedly the hemolytic streptococcus.

2. Most deaths occur in those under 5 years of age, and especially under 3 years. Halliday (1928) stated that, taking measles mortality from 5–10 years as unity, then from 0–2 years the mortality is 10–20 times greater, from 2–3 years 8–11 times greater, from 3–4 years 3½–4½ times greater, and from 4–5 years 1½–2 times more than unity.

3 Mortality is affected by a number of factors other than age Thus rickets has been incriminated as a factor tending to increase
nutrition in general is a factor. Measles devel
from other diseases is frequently fatal In New
death rate among Italian children, while thos
lowest rate. The winter fatality is about double that of the summer.

4 Perhaps the most important of these other factors is housing Halliday (1928) showed that in tenements in "slum" areas, children are exposed to infection at an earlier age than are those in new housing estates. Accordingly, in slum areas, measles is a disease of infancy, whereas in more "residential" areas school children are the main victims The children of the well-to-do tend to be infected later still, from 14–18. Many similar observations have been made. Thus in France, Debré and Joannon (1924) found that the mortality from measles under 6 years was 4 times greater in the poorest than in the richest districts Godfrey (1926), in America, found that 18 per cent. of cases and 83 per cent. of deaths occurring in the towns were in children under 3, whereas in the villages there were 11 per cent of cases and 61 per cent of deaths for this age group Picken (1921) found that the fatality rate per 100 cases in urban districts was 6 1 per cent, and in rural districts about 1 to 1 3 per cent. He showed that the incidence of the disease in one-apartment houses was 1 85 per cent, in three-apartment houses 0 69 per cent., and in larger houses 0 31 per cent. Wright and Wright (1942), investigating this problem in London, concluded that Halliday's observations seemed equally applicable. They found that statistically, substandard housing was the predominant social factor in mortality.

5. In Great Britain, the deaths from measles are now only a fraction of formerly—less than 1,000 per epidemic year The case fatality rate is about 0 2 per cent. A similar decline in the death rate has been noted in the United States, Canada, and elsewhere (see McKinnon, 1948)

McKinnon suggests that a partial explanation is the reduction in physically substandard children

Butler (1945, 1946, 1947 *a*) has discussed this problem, and it may be concluded that various factors are responsible (*a*) Greater care of the sick in the home, (*b*) hospitalization and improved treatment of severe cases, (*c*) an increased incidence on the more resistant older child as compared with earlier days, (*d*) an improved state of nutrition in childhood, (*e*) perhaps a less severe type of infection

Butler (1945, 1947 *b*) has made use of the batch fatality rate, which as far as possible relates deaths to the actual cases from which they derive. Where serial cases and deaths are recorded week by week, the batch fatality rate at the terminal week of each successive 9-weekly period is

$$\frac{\text{the sum of deaths in last 5 weeks} \times 1{,}000}{\text{Half the sum of the cases of the first 4 weeks} + \text{all the cases of the middle week} + \text{half the sum of the cases of the last 4 weeks}}$$

PATHOLOGY OF UNCOMPLICATED MEASLES

1 *Koplik's spots.* These lesions usually originate in a submucous gland, there is focal necrosis of the basal epithelium, and formation of a vesicle with exudation of

preeruptive stage, the color index being about 0 6 to 0 7 owing to a fall in hemoglobin (Schiff and Mátyás, 1918–19 *a*, Stancanelli, 1934).

During the eruptive period Kasahara and Maeda (1931–2) found an increase in reticulocytes, evidently indicative of some blood cell destruction The bleeding time was found to be shortened (Schiff and Mátyás, 1918–19 *b*).

The opsonic index has been examined, and Tunnicliff (1912) reported a decreased phagocytic activity for *Streptococcus*, *Staphylococcus*, and *Myco. tuberculosis* during the leukopenic stage. The opsonic index was also investigated by Piana (1930), who found it to be frequently lowered for *Bact. typhosum*, normal for *Br. melitensis*, and (contrary to Tunnicliff) increased for *Streptococcus*

Radiography of the chest. Radiological examination of the chest during the progress of measles was carried out by Kohn and Koiransky (1929, 1931, 1933) in a considerable number of cases Under 4 years of age 62.4 per cent. of patients showed shadows indicative of pulmonary infiltration, while over 4 years some 42.2 per cent showed these shadows. These markings were usually evident before or during the eruptive stage. Further, in the majority of cases, they found enlargement of the lymph glands in front of and below the bifurcation of the trachea This enlargement was usually maximal at the height of the eruption As these cases were unselected it shows that pulmonary involvement is a frequent occurrence in measles These results were substantially confirmed during the 1935–6 London epidemic (see *Report*, 1938).

DISTRIBUTION OF THE VIRUS IN THE INFECTED BODY

Blood. The virus probably occurs in the blood shortly before the appearance of the rash, and persists for at least 24 hours, as shown by infectivity for human volunteers, monkeys, rabbits, guinea-pigs, and eggs (Nicolle and Conseil, 1911, Anderson and Goldberger, 1911 *a*, Hektoen and Eggers, 1911, Tunnicliff, 1912, Kawamura, 1922, Degkwitz, 1927–8, Belikoff *et al.*, 1929, Taniguchi *et al*, 1935, Plotz, 1938 *a*, *b*, Rake and Shaffer, 1939, 1940, Gordon and Knighton, 1941, Hurst and Cooke, 1941, Shaffer *et al*, 1941, Rake, 1943, Konova *et al.*, 1945) Hektoen (1905) reproduced measles in a volunteer with blood taken 30 hours after the appearance of the rash Papp (1937) has claimed that the virus is associated with the leukocytes and not the plasma It should, however, be mentioned that despite the above results certain workers have found blood noninfective for monkeys, even early in the eruptive phase (Sellards, 1919 *a*, *b*, Sellards and Wentworth, 1919).

Mucous secretions. Buccal, pharyngeal, and nasal secretions are infective for monkeys, tissue cultures, and eggs in the preeruptive stage, and up to 48 hours after the appearance of the rash (Goldberger and Anderson, 1911 *a*, Anderson and Gold- … , Tunnicliff and Moody, 1922, Degkwitz, … and Shaffer, 1939, 1940, Hurst and Cooke, 1941, Rake, Shaffer, and Jones, 1941, Konova *et al*, 1945)

The eruption Mayr (1852) claimed to have infected children by nasal inoculations of scrapings from the rash Torres and Teixeria (1932 *a*) produced a rise of temperature and leukopenia in monkeys after injection with morsels of the exanthem Measles scales have been found noninfective for children (Mayr, 1860) or monkeys (Anderson and Goldberger, 1911 *b*)

MORTALITY

Measles ranks high among the main killing diseases of childhood, and this question has received attention from a number of writers (MacNalty and Wheaton, 1918, Brownlee, 1920, Halliday, 1928, Fischl, 1929, Soper, 1929, Godfrey, 1926, Degen, 1937, Brincker, 1938; Butler, 1945, 1947 *a*, *b*)

1. Most deaths occur after the first week, usually in the second or third, and are due to respiratory complications Uncomplicated measles is only rarely fatal (Wilson, 1928–9) For example, in the 1927–8 London epidemic, 86 per cent of

Koplik's spots, the nasal mucosa, and the blood monocytes. These vary in size and are found in the cytoplasm, staining with nigrosin. With regard to intranuclear bodies, Torres and Teixeria (1932 *b*) have described equatorial bands in the nuclei of the stratum granulosum and superficial malpighian layers These structures probably consist of a condensation of nuclear material.

5. *Other features.* In Degen's (1937) series of 100 autopsies certain pathological findings, scarcely to be regarded as complications, were reported Thus, the tonsils were often enlarged and inflamed, Alagna (1911) found giant cells in the tonsils in fatal cases The lymph glands were also enlarged and showed subacute inflammatory changes The spleen was frequently enlarged and congested, showing lymphoid hyperplasia The liver and kidneys were also congested and showed cloudy swelling.

It has been remarked that cases of measles coming to autopsy usually show a well-developed complication Wilson (1928–9), however, has reported a case of fatal, apparently uncomplicated, measles Changes were found only in the respiratory system, thus the lungs were congested and exuded blood-stained fluid Microscopically the alveoli were filled with clear pink-staining material and some fibrin and mononuclear cells The mucosa of the mouth, larynx, trachea, and primary bronchi showed pale and swollen epithelium, with a scanty infiltration of round cells and polynuclears. There were some areas of ulceration, beneath which were collections of mononuclear cells

EPIDEMIOLOGY

The Method of Spread of Measles

Epidemiologically it is evident that measles is infective in the prodromal catarrhal stage, and for 2–3 days of the eruptive stage Correspondingly, virus has been isolated from the upper respiratory tract in the preeruptive and early eruptive phase, it has also been found in the eruption. In some cases, infection may be spread by direct or indirect contact with the skin. Much more usually, infection is contracted by inhalation of droplets, and air-borne droplet nuclei are usually held responsible (see Wells, 1944) Larger droplets may also be responsible, as where the sufferer coughs or sneezes in the vicinity of a susceptible person Experiments of Hare and Mackenzie (1946) with *B. prodigiosus* suggest, however, that another mechanism may often be involved They suggest that exhaled particles largely fall downward and adhere to clothing Later, clouds of infective particles may be liberated by shaking the clothing Certainly there are obvious instances where infection by some such means must be invoked, and where there can be no question of droplet nuclei being responsible.

The Incidence of Measles

Age incidence The greatest number of cases of measles, and certainly the greatest number of fatal cases, occur in children under 5 years of age The great majority of adults in civilized lands has suffered from measles Thus, Butler (1912–13), investigating the measles histories of 14,000 persons, found that 78 per cent at all ages had been attacked Analyzed, these figures showed that from 0 to 4 years 8 4 per cent had been infected, while over 15 years some 97 3 per cent had suffered from measles Collins (1924) found that 90 to 95 per cent of American students had been attacked

The disease is rare in newborn infants (up to 5 months) of mothers who have had measles (Herrman, 1917, 1923, Friedjung, 1918–19) Definite cases of measles, both congenital and in the first few months of life have, however, been recorded (e g, by Ballantyne, 1893, Schulze, 1921, Ronaldson, 1926, Purves, 1934, Dyer, 1940, Lyall and Murdick, 1941) At the other extreme of life, cases have been

serum and emigration of endothelial leukocytes, the spot is surrounded by dilated vessels (Hlava, see Ewing, 1909, Denton, 1925). In certain cases the spots may present the ordinary microscopic appearance of the measles exanthem, with superficial parakeratosis or keratosis and later desquamation (Hlava). Inclusion bodies, staining with nigrosin, and occurring in the cytoplasm, have been found by Broadhurst *et al.* (1937, *vide infra*).

2 *Giant cells.* A number of authors has recorded the presence of giant cells in various tissues during the prodromal period (for a general review see Ravina and Lévy-Lang, 1937) Thus, these cells have been found in the tonsil (Finkeldey, 1931, Warthin, 1931, Mayerhofer, 1934), the appendix (references given above), the spleen, thymus, liver, and lymph glands (Hathaway, 1935; Wegelin, 1937, Semsroth, 1939, Schultze, 1943); *oral, throat, laryngeal, bronchial, and esophageal mu*cosae (Masugi and Minami, 1938; Minami, 1938, Homrighouse and McKee, 1939, Semsroth, 1939).

Warthin (1931) fully described the appearance of these cells in the tonsil Beneath the tonsillar surface epithelium is a narrow zone where there are large syn*cytial multinucleated giant cells, measuring about 100 μ. These cells are spherical* or somewhat lobed, the nuclei occurring in a central cluster, numbering up to 70–100, and being frequently pyknotic. Giant cells are also found in and between the germinal centers, and probably develop by amitotic division of lymphocyte-like [illegible] l show marked lymphoid exhaustion Similar [illegible] glands.

[illegible] res of the measles exanthem have been described by various authors (see, e.g , Catrin, 1891; Ewing, 1909, Mallory and Medlar, 1920). Ewing gives a full bibliography. Mallory and Medlar's findings will be described, as these seem to represent the normal.

[illegible] nall [illegible] the epidermis with endothelial leukocytes which may collect to form pustules under the cornified layer, these pustules soon dry up to form a plaque (Ewing's hyper*keratosis*) *Retrograde changes appear as necrosis of epithelial cells which* affects individual or groups of cells Necrosis takes place also in the hair sheaths and sebaceous glands, cells in these areas show cytoplasmic droplets of varying size and shape.

The corium also shows changes, in the superficial layers of the corium and papillae there is an active exudate of endothelial leukocytes, which have mostly developed by proliferation and emigration of vascular endothelium. The cytoplasm of these cells is basophilic, shows a network, and often contains polymorphs or leukocytes. The vascular endothelium in these areas is swollen and the cytoplasm granular. The measles lesion probably starts in and around the superficial vessels of the corium, emigration of endothelial leukocytes occurs and spreads to the overlying epidermis, then small pustules may form which later dry up.

4. *Inclusion bodies* A variety of inclusion bodies has been described in measles, thus Catrin (1891) found large mulberry-shaped masses in the cytoplasm of the epidermal cells, pushing the nucleus to one side. Somewhat similar bodies were found by Ewing (1909), these being irregular, more or less homogeneous basophilic rings, spheres, or crescents occurring in the cytoplasm of the malpighian cells. The bodies described by Catrin and Ewing are probably of degenerative nature Small [illegible] ascular endothelium by Mallory and Medlar [illegible] ively and may have represented phagocyted [illegible] ody-like granules have been recorded by Lipschutz (1926), [illegible] *itrophyten* They measured 0.2 to 0 3 μ in diameter and were found in epidermal cells

Broadhurst *et al.* (1937, 1938) have found granular inclusions in the cells of

inter-epidemic periods in which few cases are recorded. In large countries, composed of numerous units, there is usually an epidemic in progress somewhere or other. In this case compensation between the occurrence of endemic cases and epidemics renders the morbidity and mortality curves more uniform. However, the state of affairs is not always so, because variations in the curves may be quite considerable. In England in the years 1919–30 the maximum death rate recorded was 3½ times the minimum, and in Germany the figure was 5 times In countries with a smaller population, as already mentioned, the variations were greater. Thus in Scotland and Denmark the maximum rate was 17 times the minimum, and in Switzerland 23 times as great, exceptions to the general rule did occur, and in Belgium, Spain, and the Netherlands there were only small variations recorded

Examinations of the mortality curves for the whole decade showed a tendency for a phase of low mortality to succeed immediately one of high mortality. For example, in Germany in 1923 the mortality rate was 13 4 and 2 6 in 1924, in England in 1920, 19 2, in 1921, 5 9, in Sweden 9 1 in 1924 and 1 1 in 1925; and so forth

Measles in tropical lands generally presents similar features to those found in more temperate climates. The disease may assume serious proportions among workers on tea estates and other plantations where isolation is virtually impossible (Kingsbury, 1928, Manson, 1935). Kingsbury has pointed out that children may suffer from bronchitis probably due to the passage of ankylostomes and ascarides through the lungs.

Estimates of the Number of Susceptibles

Various mathematical investigations have shown that it is possible to determine the precise number of susceptibles in any one community (Hamer, 1906, Brownlee, 1908–9, Stocks and Karn, 1928, Hedrich, 1930, 1933). In estimating the number of susceptibles it must be taken into account that a number of children become temporarily immune due to latent infection. This fact has been largely established by Stocks, who has shown that about 4 to 5 times as many children as suffer from actual attacks acquire immunization sufficient to protect them for one winter (Stocks and Karn, 1928, Stocks, 1930, 1933).

Periodicity of Measles Epidemics

It is well known that measles epidemics in any one area tend to recur regularly. The interval between epidemics, or as it is called the "period," is usually approximately 2 years. This question of periodicity has been studied by a number of authors (e g, Hamer, 1906, MacNalty and Wheaton, 1918, Brownlee, 1916–18, 1918–19, 1919, Picken, 1921, Soper, 1929, Petersen, 1940) Greenwood (1935) gave an excellent review of the subject. Butler (1946, 1947 *b*) describes a 2-year periodicity in Great Britain of recent years There is a small increase in incidence from January to July, with a partial decrease in the autumn The main incidence is in November to March There is only a short interval between the end of one cycle and the beginning of the next The partial evacuation of children from London at the beginning of the second world war led to a postponement of the 1940 epidemic (Martin, 1942, Stocks, 1942, Swyer, 1942). The presence of periodicity is undoubted, but to explain it is more difficult

1. The older view, enunciated by Hamer (1906), conceived the progress of a measles epidemic as a relatively simple affair. At the start each case gives rise to several more cases, and the curve ascends steeply Then a critical period is reached and eventually the epidemic declines as each case does not invariably give rise to another. This explanation was further elaborated by Soper (1929) to include the effect of seasonal variation

2. This theory supposes that periodicity is largely due to an alternating increase and decrease in susceptibles Observations on measles in London from 1900 to 1918

recorded in patients aged 57 (Hall, 1930), 71 (Frewin, 1931), and 76 (Graham, 1930).

Seasonal incidence. In his work on periodicity in measles (*vide infra*), Brownlee discussed the question of seasonal periodicity. Thus he found there were usually 2 maxima of incidence, in winter and in spring In London, he found the highest incidence in December and in April or May. In Edinburgh, Bristol, Birmingham, Sheffield, and Manchester the spring outbreak was the major one. In Newcastle, Liverpool, Glasgow, and Salford the 2 outbreaks were of equal importance. London suffered from an annual spring outbreak and Brownlee suggested that this was due to the prevalence of an epidemic with a strictly annual period Hindman and Harmon (1934) have also investigated the seasonal incidence of measles in America

In Europe, from 1926 to 1931, no less than 92 per cent. of the annual maximal periods of morbidity occurred from December to May (*League of Nations Epidemiological Reports*, 1932). Although in Europe the maximal morbidity and mortality occurred in the winter and spring, in Spain, Portugal, and Egypt it was in the hot weather. In contrast the maxima occurred in the cold weather in Algeria, Turkey, Palestine, and Iraq.

Influence of Degree of Exposure

Measles is, of course, a highly infective disease, but it stands to reason that the more intimate the degree of exposure the more certain is the infection to develop (see, e.g, Aycock, 1934, Karelitz and Schick, 1935). Discussing the work of Top (1937) and personal observations, Karelitz and Karelitz (1938) concluded that the incidence of measles rises with the intensity and duration of exposure to the source of infection, for periods of exposure up to about 8 days. Thereafter, increased exposure does not appear to raise the incidence.

There is no doubt, from these recent and many other studies, that the most intimate type of exposure occurs in the home, and not in schools, wards, vehicles, or at parties (see in particular, Karelitz and Karelitz, 1938). In conformity with this view, it is generally easier to secure protection with antiserum *in the hygienic* conditions of a hospital ward than it is in the private house (Karelitz and Schick, 1935) In further support of the importance of home exposure, with regard to overcrowding and housing conditions, Halliday (1928) found that the slum child tended to be exposed to infection earlier than well-to-do children, while Picken (1921) showed that the incidence of measles in one-apartment houses was about 6 times that in larger houses (see also section on Mortality) Stillerman and Thalheimer (1944) found that the secondary attack rate in 200 intimately exposed susceptible family contacts aged 1 month to 14 years was 75 per cent. The attack rate was lowest for children in early infancy, highest for those aged 1–7 years (80–90 per cent), and only 15 per cent for those 10–14

Geographical Distribution of Measles

Measles has been endemic in most parts of the world for many years. In certain isolated communities, however, infection has only occurred at long intervals, usually due to the arrival of a case from an infected ship (*vide infra*). The bulk of the following information is summarized from the special report of the League of Nations on the World Distribution of Measles from 1919 to 1931 (*League of Nations Epidemiological Reports*, 1932)

Measles differs from many other contagious diseases in that it does not evolve synchronously in neighboring countries Thus one cannot say that such-and-such a year was a "measles year" throughout the world, each country functions as a separate unit In any given country the morbidity and mortality curves may be very erratic, the variations being greater in the smaller countries This is because the smaller countries comprise epidemiological units in themselves, roughly comparable to a single city of a larger country. In the small country there will be

to 25. There were 11,000 cases in Tonga following the arrival of a ship with a case on board (Carrick, 1939).

Hawaii was visited by a very severe outbreak in 1936–7 (see *Public Health Report*, 1937) The mortality was extremely high: 102 per 100,000 The population was thought to be particularly susceptible as measles had been infrequent for the previous 5 years

Faroes. Measles was prevalent in the Faroes in 1781, but one of the most classical of all these types of outbreak occurred in 1846, and was described by Panum (1847, 1851).[1] Within 6 months 6,000 out of the 7,782 inhabitants had become infected Those old persons who had had measles in 1781 were immune, whereas contemporaries who had escaped at that time now contracted it The case mortality was low, only 1.7 per cent., although the morbidity figure was so high. Staermose and Kofoed (1938) described an outbreak in adults following a meeting to obtain whale flesh.

Other outbreaks. Numerous other outbreaks of a similar nature have been recorded (see Hirsch, 1883) in India, China, Japan, Egypt, Abyssinia, Tunis, West Coast of Africa, Australia, New Zealand, and other places. A particularly severe outbreak involved the Hudson Bay Indians, and another the Amazon natives in 1749

The Control of Measles

Measles attacks the great majority of persons, and the bulk of the mortality occurs in children in the first few years of life. What can be done to deal with the situation? The following measures have been suggested by various authorities (e g, Brownlee, 1920, Halliday, 1928, Ruhland and Silverman, 1928; Stocks, 1931, Gunn, 1942)

1. Special attention should be paid to children under 3 years of age. During a measles epidemic they should not attend any meeting places or have undue intercourse with other children Contacts may be sent away to houses in the country or less crowded surroundings where there is less chance of disseminating the infection.

2. Quarantine need not be strictly enforced for the first week after exposure, but should continue thereafter for 10 to 14 days.

3. Weakly children exposed to infection should be injected with convalescent serum early in the incubation period In the case of other, more robust, children, sero-attenuation should be aimed at in every case (*vide infra*).

4 In all cases particular attention should be paid to early diagnosis Parents, school teachers, and all youth workers should be well instructed in the prodromal manifestations of measles Children developing catarrhal symptoms must be isolated at once

5. Extra home-nursing as well as medical facilities should be freely available. There is an increasing tendency to urge hospitalization, especially of the poorer classes (see Brincker, 1936)

6. It has been suggested that children should be actively immunized by inoculation of virus on the nasal mucosa, a "covering" dose of convalescent serum being given later (*vide infra*)

By widespread adoption of such methods it is probable that measles could be largely prevented, indeed Stocks (1931) has stated that theoretically measles could be completely eradicated To the adoption of such regulations in Syracuse (U S A.) Ruhland and Silverman (1928) attributed a fall in the case fatality rate for children under 3 years from 5 1 per cent to 1 6 per cent. Certain methods will be discussed further

[1] Appreciations of Panum's important observations as well as English translations of extracts from his papers have appeared (Little, 1931, Gafafer, 1935–6).

may be quoted to illustrate this theory. Thus, measles did not arise in a community until the number of susceptibles rose to 25 per cent. Once the epidemic began it did not flare up at once, but rather it gathered speed slowly, waiting perhaps for a suitable combination of factors The epidemic did not cease till the number of susceptibles fell to 20 per cent. (Brincker, 1936). Continuing the cycle, with the passage of time, susceptibles began to increase again, as more children were born, while those not previously attacked remained susceptible. Eventually there arose a high enough percentage of susceptibles and a further epidemic occurred.

It has already been mentioned briefly that a certain number of persons acquire immunity by latent immunization. Although they do not suffer from any obvious manifestations of the disease, they none the less become immune to it. This concept has followed largely from the work of Stocks and Karn (1928), who examined the *measles statistics in the London borough of* St. Pancras from 1916 to 1918 (see also Stocks, 1930). They followed the measles histories of children in homes in which there had been cases, and contrasted them with children from noninfected homes. They found that there was a definitely lower incidence of measles in those children known to have been exposed to infection. The other children also appeared to have acquired some immunity, but this did not last so long, probably for about 18 months. Stock [illegible]
don boroughs of [illegible]

There is thus [illegible]
tions in immunity. During an epidemic there is a great increase in immunes, composed partly of recovered cases and partly of those becoming latently immunized. After the epidemic the latent immunity passes off and newborn children help to swell the increasing number of susceptibles When the number is high enough a further outbreak arises and the cycle proceeds as before

3 Brownlee attributed periodicity to the biological activity of the infecting organism—alternate phases of quiescence and activity being postulated

Conclusions.

The most characteristic feature of a measles epidemic is its periodicity, but it must be confessed that the mechanism responsible for this is somewhat obscure. Brownlee attributed the phenomenon mainly to a factor inherent in the organism Stocks and Karn, on the other hand, think that the phenomenon of fluctuations in immunity, mainly dependent on latent immunization, is the most likely explanation. Greenwood (1935) in his discussion of this subject inclines to the opinion that immunity mechanisms play the predominant rôle.

Measles Epidemics in Virgin Soil

Measles is endemic in most of the congested parts of the world, but is often absent in isolated communities Certain of these communities, especially island ones, have suffered from time to time in a characteristic fashion Measles has usually been introduced by an infected person on board ship In these outbreaks all persons have been susceptible, irrespective of age, the attack rate has been very high and there has often been a high percentage of fatalities Certain of the best known of such visitations will now be described, but those who desire further information should consult the comprehensive article of Hirsch (1883)

Pacific Islands. A severe outbreak of measles occurred in Fiji in 1875, when approximately 25 per cent of the natives died (Corney, 1883–4) In 1911 the island of Routumá was visited by measles following the call of a ship with a case on board (MacDonald, 1912, Corney, 1912–13) There had been no previous outbreak in this island for at least a generation The death rate figure reached 246 5 per 1,000, the majority of deaths occurred in young children and in adults aged 20

ultraviolet light. Some success seems to have attended this work (Wells, Wells, and Wilder, 1942, Wells, 1945).

Education of parents.

This necessary duty has been repeatedly stressed Health authorities should instruct parents that measles in children under 3 is a very dangerous disease. If the attack can be postponed till a later age the child is more likely to survive. Parents of children under 5 should keep them at home during measles epidemics

Nursing and hospitalization.

Probably home treatment is perfectly satisfactory provided a district or other nurse can attend regularly. If this is not possible, or the house very unsuitable, hospitalization should be carried out As has been said, there is an increasing tendency to effect hospitalization, many feel that in this way complications can be recognized more readily and treated more efficiently. However, recent observations on the frequency of cross infection in open measles wards (see above) point to the necessity for discrimination in urging hospitalization Cruickshank (1943), for instance, suggests that children should be sent only if home conditions are poor. Wright (1945) makes the following recommendations for nursing measles cases in hospital, to reduce the risk of cross infection (1) There should be 12 feet between beds (2) Infants under 1 should have a priority on isolation accommodation (3) Patients with suspected streptococcal infection should be isolated (4) Patients must not be moved directly from one open ward to another. (5) Dust-suppressive measures should be taken, including the use of oil (6) Individual equipment must be used for nasal toilets, and nurses must wash their hands after each treatment. (7) Whenever possible, fomites should be sterilized between each usage. (8) Barrier nursing should be used for any patient developing intercurrent respiratory, gastro-intestinal, or skin infection (9) Nurses should wear masks

It seems probable that sulfadiazine administered as a routine to all children in the ward may reduce the incidence of cross infection (Begg, Smellie, and Wright, 1947).

Measles Outbreaks in Hospitals and Children's Institutions

To obviate quarantine Steinholz (1933) has recommended the following scheme.

1. The primary case is transferred to a fever hospital.
2. Each susceptible in the ward is given 40 c c. of parental whole blood
3. Any new cases, certified to have had measles, may be admitted
4. Other entrants receive 40 c c. of blood
5. All cases that have had measles may be discharged when the time arrives
6. Cases that have been injected with whole blood may not be discharged until at least 14 days have elapsed By such means Steinholz claims to be able to keep a ward open and in working order

Crosbie (1938) has also reported on the successful application of a similar method He removes the case and immunizes all contacts with adult serum or globulin (placental) The ward need not be closed if all entrants have had measles The Medical Research Council in Great Britain (1944) recommends 2 alternatives (*a*) to protect all susceptibles with convalescent serum and to continue to admit, or (*b*) to protect all children under 3, and older debilitated children, and to secure attenuation in older robust children, the ward being quarantined for 21 days

Notification.

It is questionable how far notification is of value in restricting the spread of infection, but some believe that it has this effect Thus Boyd (1928), discussing the value of reporting in Pretoria, attributes a fall in death rate from 1.38 per 1,000 in 1907–12 to 0 26 in 1922–7 as due partly to the increased control of cases and contacts rendered possible by notification As in other infections, probably many cases are never reported. For example, in the United States, Sydenstricker and Hedric (1929) found that only 25 8 per cent of the entire incidence was reported

Measles in schools.

The question of measles in schools is one of great importance, for it is here, indubitably, that the majority of children contract their infection

Boarding schools A special committee of the Medical Research Council (*Report*, 1938) found that measles is more likely to spread than any other infective fever, and they recorded an attack rate on susceptibles of up to 88 per cent.

Day schools. During a measles epidemic practically every day school will be infected, and become the main disseminator of infection.

Nursery schools. It has been shown that children attending day nurseries are much more likely to develop measles than children who stay at home. They tend further to develop it at an earlier stage and more especially in the winter—both serious points as regards mortality (McLaughlin, 1947)

Exclusion from school The official regulations of Great Britain, issued jointly by the Ministry of Health and the Board of Education (*Memorandum*, 1942), can be summarized as follows

1 Children attacked by measles should be kept from school for 2 weeks from the first appearance of the rash.

2. As regards contacts, infants who have not had the disease should be excluded for 14 days from the date of the appearance of the rash in the last case in the house Other contacts can attend Any contact with a cough, cold, chill, or red eyes should at once be excluded

Forbes of Brighton consistently criticized the value of exclusion of home contacts (1930, 1933, 1936, 1939) He states that home contacts attending infected schools should not be excluded, although the contacts attending uninfected schools should be Exclusion during the long incubation period is useless, while exclusion toward the end of this period allows the child to run about in and near his home infecting infants, as prodromal catarrh is seldom regarded by the parents as adequate grounds for keeping the child indoors Whereas, Forbes states, if the child attends school his prodromal symptoms will be recognized at once and the child sent home out of harm's way

Forbes, however, urges the exclusion of "under fives" from infected schools, or from noninfected schools if weakly, or living in unhealthy surroundings (see, e.g, 1933)

In Brighton, since 1923, home contacts attending infected schools have not been excluded Home contacts attending uninfected schools are excluded, however These procedures result in a minimum loss of school attendance

School closure School closure usually results in a slowing-down of the epidemic but it flares up again on reopening It may, however, be of use in the prevention of respiratory fatalities during very inclement weather (Forbes, 1930, 1933, 1936) The official regulations state that school closure is generally useless, but in country districts where the children have to come some distance it may prove an effective measure Crèches should be closed during a measles epidemic (Forbes, 1930)

Ultraviolet irradiation On the assumption that measles is largely spread by droplet nuclei, experiments have been carried out by irradiating classrooms with

rash on intradermal injection of susceptibles, and can be neutralized by measles convalescent serum (Ferry and Fisher, 1926). Measles convalescent serum agglutinates the organism (Ferry and Fisher, 1926, Ferry, 1929). Antitoxin produced in animals was said to give protection against measles (Ferry, 1929, Ferry *et al*, 1928)

[illegible]

staphylococci, diphtheroids, and influenza bacilli are all mentioned as having been found in measles cases (see Thomson and Thomson, 1931).

Nonconfirmatory work. The impression will have been gained that there is, apparently, no one specific measles bacterium, for a variety of different cocci and other organisms has been claimed as etiological by their respective discoverers The diversity of types of "measles cocci" was stressed by Park *et al* (1927) who isolated a great variety from cases of measles Ferry and Noble (1929) also noted the wide range of variation among the various "measles cocci." Park *et al.* were unable to differentiate between immunes and nonimmunes by intracutaneous injection of toxins prepared from "measles cocci" (see also Smith and Fraser, 1928–9) Bradford (1929) also found the skin test valueless as a means of distinguishing between immunes and susceptibles, using Tunnicliff's coccus and *S. morbilli*. Various workers have failed to isolate green-producing cocci from the blood (see, e g., Long and Cornwell, 1927) Other workers have isolated "measles cocci" from the pharynx of measles cases but no more commonly than from healthy persons Thus Smith (1928–9) found no evidence that these cocci were other than part of the normal flora of the upper respiratory tract, and the majority of bacteriologists agrees with this view It is evident, therefore, that there is no substantial basis for believing that measles is caused by cultivable bacteria. We shall now describe the properties of the virus believed to be the true causal agent.

THE VIRUS OF MEASLES

Measles was probably first shown to be an infective disease by Mayr (1852), and later by Hektoen (1905), who employed human inoculation experiments. Josias (1898), one of the first to attempt animal transmission, inoculated monkeys with measles secretions and obtained a slight rise of temperature and a rash However, little advance was made till 1911 when a number of investigators reported the susceptibility of the monkey In the same year the etiological agent was shown to pass a Berkefeld filter by Goldberger and Anderson (1911 *b*), thus definitely establishing measles as a virus disease.

Human Inoculation Experiments

The work of Home (1759) is always quoted in this respect He inoculated some 15 children with measles blood by scarification, the majority developed a rash and pyrexia, but within an incubation period of 10 days Rigid precautions to exclude outside infection do not seem to have been made Home's work was pioneer and therefore of great value, but as his experiments were scarcely sufficiently controlled it is not possible to accept that he actually transmitted the infection Mayr (1852) infected susceptibles by inoculating them intranasally with nasal secretion from measles cases He also produced a mild attack by scarifying the skin with swabbings from a measles lesion. A number of inoculation experiments of a doubtful nature was carried out between the time of Home and that of Hektoen (1905) These have been reviewed by Hektoen and also by Kato (1928)

Hektoen (1905) withdrew blood from a case of measles on the fourth day and inoculated 4 c c. into ascites broth, after 24 hours the mixture was injected subcutaneously into a human volunteer, who had been quarantined This patient developed a transient pyrexia after 14 days From another case Hektoen withdrew

CHAPTER XXI

THE VIRUS OF MEASLES

RÔLE OF CULTIVABLE BACTERIA

VARIOUS bacteria have been claimed as the cause of measles, but it is now generally believed that measles is a virus disease, and that these organisms are accidental contaminants or perhaps secondary invaders. Those interested in the question of the cultivable "measles bacteria" should consult the exhaustive monographs published by Thomson (1924,[1] 1926, Thomson and Thomson, 1931). These organisms will now be discussed separately.

Tunnicliff's coccus. Perhaps the most extensive work on "measles cocci" has been carried out by Tunnicliff and coworkers and their main findings may be summarized as follows·

(*a*) A diplococcus, *Diplococcus morbillorum*, occurs in the blood of measles cases, during the preeruptive and eruptive stages, as well as in the throat, eyes, and nose. This organism grows, at first anaerobically, in semicoagulated horse serum and whole blood ascitic dextrose agar shakes; later, aerobic growth occurs. The organism can be subcultured on blood agar and produces green or α-hemolysis A filtrable exotoxin is produced (Tunnicliff, 1917, 1918).

(*b*) Measles diplococci are immunologically distinct from nearly all similar cocci, but lose their specificity by growing in immune horse serum, or at 40° C. (Tunnicliff, 1927, 1933).

(*c*) The organism is filtrable through a Berkefeld N filter when occurring in exudates, but not in cultures (Duval and Luzenberg, 1932–3).

(*d*) Convalescent measles serum and diplococci fix complement (Tunnicliff and Brown, 1918). Convalescent serum agglutinates diplococci (Tunnicliff, 1919) Placental extract and convalescent serum have a high opsonic effect on the diplococci (Tunnicliff, 1936)

(*e*) Rabbits injected with diplococci show a rise of temperature, Koplik's spots, and an eruption.

(*f*) Injected rabbits show the development of specific serum opsonins and agglutinins (Tunnicliff and Brown, 1918)

(*g*) A goat serum with antitoxic and antibacterial powers was produced by injection of diplococci and filtrates This was said to have a prophylactic effect in children exposed to infection (Tunnicliff and Hoyne, 1926 *a*, *b*).

(*h*) This goat serum protected rabbits against injections of infective measles material (Tunnicliff and Hoyne, 1926 *b*)

(*i*) Diplococcal cultures and filtrates produce a skin rash in susceptibles, but not in persons who have had measles This toxin is neutralized by convalescent measles serum and goat serum (Tunnicliff, 1925, Tunnicliff and Taylor, 1926)

Caronia's coccus Caronia isolated a small gram-negative diplococcus from the blood and secretions of measles cases, during the rash and prodromally (see, e g, 1923). This organism grew anaerobically in Tarozzi-Noguchi's medium Convalescent serum showed agglutination and opsonization of these cocci The injection of rabbits with cocci produced a measles-like infection Meyer (1925–6), however, isolated similar cocci from control material

Ferry's coccus The name of *Streptococcus morbilli* has been given to a streptococcus isolated from the blood of measles cases by Ferry and Fisher (1926) The organism is gram-positive and grows aerobically It produces a toxin which gives a

[1] References are appended at the conclusion of Ch XXII, p 235 et seq

(*a*) A rise of temperature may occur after 8 to 11 days, the temperature may remain raised, be remittent, or soon fall.

(*b*) Catarrh affecting the nose and eyes develops in about 50 per cent.

(*c*) About 3 to 4 days after the fever a discrete roseolar papular rash develops, especially on the face and chin and spreading to the neck, chest, abdomen, upper arms, and thighs (Blake and Trask, 1921 *a*, *b*). Brown scaling occurs later

(*d*) Koplik's spots may be seen as discrete red spots on the inside of the lips (Lucas and Prizer, 1912, Kawamura, 1922, Blake and Trask, 1921 *a*, *b*).

(*e*) In the preeruptive stage a leukopenia may develop, although shortly after inoculation there is a leukocytosis (Hektoen and Eggers, 1911, Lucas and Prizer, 1912, Tunnicliff, 1912, Blake and Trask, 1921 *a*, *b*, Torres and Teixeria, 1932 *a*) In the leukopenic stage Tunnicliff (1912) found that there was a decided decrease in the phagocytic activity of the leukocytes

(*f*) Hurst and Cooke (1941) could not pass the infection in series for more than 4–6 transfers

The distribution of the virus in infected monkeys The virus must be present in the catarrhal secretions as infection has been spread by contact (Goldberger and Anderson, 1911 *a*). The virus is present in the buccal mucosa of infected monkeys killed 2 to 6 days from the onset (Blake and Trask, 1921 *a*). The virus is found in the blood 24 hours before the appearance of Koplik's spots and persists for 36 hours after the appearance of the rash (Lucas and Prizer, 1912) Confirming these findings, Blake and Trask (1921 *a*) found monkey blood infective for the first 5 days of the illness Plotz (1938 *b*) found that virus might persist in the blood of inoculated animals in the absence of symptoms

Pathology The histological features of the skin rash have been studied by Blake and Trask (1921 *b*) as well as by Lipschutz (1928). The corium shows multiplication of the endothelial leukocytes with extravascular emigration The cyto-

epidermis shows

with the eventual

rosis of epithelial

(1928) has found

(*vide supra*)

The mucosa of the lips presents a similar picture with activity of the endothelial leukocytes in the deeper layers, this reaction is also evident beneath the stratified epithelium where the cells collect in small foci. Vacuolation and necrosis of the epithelial cells occur also. It will be noted that the features described in the skin and labial mucosa are very similar to those found in human lesions Giant cells have been described in lymph nodes (Gordon and Knighton, 1941)

Rabbits.

A number of authors has reported on the susceptibility of rabbits (Nevin and Bittman, 1921, Harde, 1921, Duval and D'Aunoy, 1922 *b*, *c*, Tunnicliff and Moody, 1922, Grund, 1922, Caronia, 1923, Scott and Simon, 1924 *a*, *b*, 1925, Hibbard and Duval, 1925–6, Belikoff, Dwijkoff, and Truschina, 1929, Baar and Benedict, 1931, Taniguchi *et al*, 1935)

Rabbits can be injected intravenously or intratracheally, and various reactions may develop following an incubation period of 2 to 5 days

(*a*) Pyrexia

(*b*) Many have recorded leukopenia, although carefully controlled work by Purdy (1925) did not show any such reaction

(*c*) Coryza, conjunctivitis, and upper respiratory tract inflammation

(*d*) Koplik's spots

(*e*) A *maculopapular rash develops on the face, chest, neck*, and abdomen

(*f*) Orchitis may rarely follow an intravenous injection (Taniguchi *et al*, 1935) Post mortem, nephritis may be found

blood 30 hours after the appearance of the rash. After being placed in broth, the blood was injected subcutaneously into a patient. On the eleventh day pyrexia, cough, conjunctivitis, and tonsillitis developed, while on the fourteenth there was a rash. There seems to be little doubt that Hektoen was one of the first to demonstrate the infectivity of measles in a convincing manner. It should be added, however, that Sellards (*1919 a, b*) failed on many occasions to transmit the infection by injections of human blood.

Adducing further evidence on the infectivity of blood, Bauguess (*1924*) reported that measles had been conveyed in the course of a blood transfusion from a donor in the prodromal stage of measles. Degkwitz (*1927*) reported that measles could be reproduced in man by inoculations of Berkefeld-filtered blood, and filtrates of nasal secretions. Taniguchi's virus, maintained by rabbit testicular passage, produced localized papules and vesicles on intradermal injection and generalization sometimes occurred (Taniguchi *et al*, *1935*). Later workers have demonstrated the infectivity of measles blood for children (Shaffer *et al.*, *1941*, Rake, *1943*).

Children have been infected with virus cultivated on the chorio-allantois, following nasal instillation, inhalation, intradermal or subcutaneous injection. The intradermal and inhalation methods of introduction of virus secure infection in the majority, but nasal instillation is less certain. The symptoms are usually mild, and the infection is characterized by Koplik's spots, rash, fever, conjunctivitis, coryza, and leukopenia (Shaffer *et al*, *1941*; Rake, *1943*, Stokes *et al.*, *1943*).

Animal Experiments

Monkeys.

Numerous investigators have shown the monkey to be susceptible to experimental measles (Nicolle and Conseil, *1911*, Anderson and Goldberger, *1911 a, b*, Goldberger and Anderson, *1911 a, b*, Hektoen and Eggers, *1911*; Lucas and Prizer, *1912*, Tunnicliff, *1912*, Blake and Trask, *1921 a, b, c*, Nevin and Bittman, *1921*, Tunnicliff and Moody, *1922*, Purdy, *1925*; Hibbard and Duval, *1925–6*, Degkwitz, *1927*, *1927–8*, Lipschutz, *1928*, Torres and Teixeria, *1932 a*; Taniguchi *et al*, *1935*, Plotz, *1938 a, b*, Rake and Shaffer, *1939*, *1940*, Shaffer *et al.*, *1941*; Gordon and Knighton, *1941*, Hurst and Cooke, *1941*, Shaffer, Rake, and Hodes, *1942*, Konowa *et al*, *1945*).

The following species of monkey have been found susceptible *Macacus sinicus* (the bonnet monkey), used by Nicolle and Conseil (*1911*), cynomologus and rhesus monkeys (Goldberger and Anderson, *1911 a*), and *Macacus fuscatus* (Kawamura, *1922*) Hurst and Cooke (*1941*) found the following susceptible. *M. mulatta*, *M radialus*, and *Cebus fatuellus* The following were negative· *M. irus*, *M. maurus*, and *Cynopithecus niger*. The inoculum in these experiments has often been human blood, mixed catarrhal secretions have also been used, as well as preparations of the exanthem Monkeys are susceptible to virus grown in the egg or tissue culture and develop symptoms similar to those found by workers using human material (Rake and Shaffer, *1939*, *1940*, Rake, Shaffer, and Jones, *1941*, Shaffer *et al.*, *1941*, Rake, *1943*)

Routes of inoculation. Monkeys can be infected by a variety of routes combined intracerebrally and intraperitoneally (Anderson and Goldberger, *1911 a*), by contact between a healthy monkey and one in the catarrhal stage (Goldberger and Anderson, *1911 a*), subcutaneously (Goldberger and Anderson, *1911 a*, Kawamura, *1922*), by spraying and swabbing the mouth and throat with catarrhal secretions (Goldberger and Anderson, *1911 a*), intravenously (Blake and Trask, *1921 b*), intratracheally (Blake and Trask, *1921 a, b, c*), intranasally Hurst and Cooke (*1941*) found splenectomized monkeys were more susceptible.

Course Following injection there is an incubation period of 6 to 11 days after which a variety of symptoms may develop (see in particular, Blake and Trask, *1921 a, b*)

on human injection (Degkwitz, 1927). Ueda (1937) also reported cultivation of the virus by the hanging-drop method. Plotz (1938 *a*) claims to have cultivated the virus from human blood by growth in a chick embryo, Tyrode, serum medium, tenth passage material provoked a typical reaction on inoculation in a monkey. Rake, Shaffer, and Jones (1941) also propagated virus in tissue cultures.

Pocks were produced on the chorio-allantois after inoculation of the egg with material from a measles skin lesion (Torres and Teixeria, 1935). Microscopically, the pocks consisted of nodular ectodermal proliferations, polymorphic infiltrations were found in the mesoderm.

The virus has been grown on the chorio-allantois from blood removed in the preeruptive stage (Wenckebach and Kunert, 1937). Degkwitz and Mayer (1937) have also reported growth on the chorio-allantois. Taniguchi's virus also grows on the chorio-allantois (Taniguchi *et al*, 1935).

These earlier observations have been abundantly confirmed of recent years and chorio-allantoic virus has been used in human immunization experiments (Heinzmann, 1939, Rake and Shaffer, 1939, 1940, Mayer, 1941, Rake, Shaffer, and Jones, 1941, Maris *et al*, 1943, Rake, 1943; Stokes *et al*, 1943).

Muller (1942), however, obtained negative results. Hurst and Cooke (1941) could not infect by the amniotic route.

Resistance to Physical and Chemical Agents

The virus is destroyed at 55° C. in a quarter of an hour (Goldberger and Anderson, 1911 *b*). It can be preserved at 0° C. for several days, or at −35° C. for 4 weeks (Rake and Shaffer, 1940). Tissue dried *in vacuo* from the frozen state retained virulence for several months (Hurst and Cooke, 1941).

Degkwitz (1927) found that the virus survived for some weeks if blood was drawn off in the early eruptive stage and diluted 1 7 to 1.10 with saline, and kept at 0° C

The virus resists 50 per cent. glycerol for 3 months (Taniguchi *et al*, 1935).

The virus resists 10 per cent. ether at room temperature for 30–40 minutes (Rake and Shaffer, 1940; Rake, Shaffer, and Jones, 1941).

Relationship to Other Viruses

It is generally agreed that the virus of measles is quite distinct from other virus agents. Thus Taniguchi showed that his virus could be absolutely separated (by viricidal tests) from vaccinia, varicella, herpes, or virus III (Taniguchi *et al*, 1935).

(g) Harde (1921) reported that if the blood of measles cases was injected intravenously and the skin of the rabbit shaved, an erythema developed 48 hours later and lasted for 24 to 48 hours.

Taniguchi *et al.* (1935) reported that their virus could be transmitted by the cornea, anterior chamber, and rarely, by intracerebral injection; following intracutaneous injection a local eruption developed. After intratesticular injection an acute orchitis developed (see also Ueda, 1937).

Other workers have failed to infect rabbits (Gordon and Knighton, 1941, Hurst and Cooke, 1941).

Guinea-pigs.

These animals have also been found susceptible (Duval and D'Aunoy, 1922 *a, c*, Tunnicliff and Moody, 1922; Hibbard and Duval, 1925–6; Kawakubo, 1932, Taniguchi *et al.*, 1935; Ueda, 1937; Konov, 1943). When injected intracardially or intratracheally the following symptoms may develop after 9 to 12 days. rise in temperature, leukopenia; at postmortem an intense hemorrhagic nephritis may be found. Animals can also be infected intratesticularly when fever and leukopenia develop with local swelling (Kawakubo, 1932, Taniguchi *et al*, 1935, Ueda, 1937, Marotta, 1939 *a*), microscopically there is atrophy of the seminiferous tubules with an interstitial infiltration of histiocytes and mononuclears In addition to orchitis an interstitial pneumonia is found, and there may be cellular infiltrations in the liver, kidneys, and heart Taniguchi *et al*, (1935) further reported that guinea-pigs could be infected corneally and intracutaneously. Gordon and Knighton (1941) were unable to infect these animals

Other animals.

Mice can be infected corneally and intracerebrally, and fowls and pigeons intracutaneously (Taniguchi *et al*, 1935). Gavrilov (1939) also claimed to have infected mice. Rats could not be infected (Gordon and Knighton, 1941)

Morphology and Filtrability

Elementary bodies (0 25 μ diameter) were found in preparations made from the corneal epithelium of the rabbit (Taniguchi *et al*, 1935) Elementary bodies (slightly larger than Paschen bodies) were found by Coles (1937) in the blood, nasal, throat, and lacrimal secretions

Various types of inclusion body have been described in the skin lesions (*vide supra*), some of these are probably purely degenerative such as the mulberry, ring, and crescentic forms Structures which may represent the etiological agent are those described by Lipschutz (1928, 1929) They are small intracytoplasmic basophilic granules found in the epidermal cells Further study is required, however, before the precise nature of these bodies can be ascertained Broadhurst *et al* (1937, 1938) have recently reported small granular inclusions in human blood monocytes, Koplik's spots, and the nasal mucosa These inclusions develop also if blood cells are grown in tissue culture The cell may break down, liberating small granular elementary bodies Growths of fibroblasts can be infected with measles virus, inclusions developing in the newly formed cells. The bodies are best stained by nigrosin.

The virus is filtrable through the following filters Berkefeld N (Goldberger and Anderson, 1911 *b*; Degkwitz, 1927, 1927–8, Blake and Trask, 1921 *a*, Taniguchi *et al*, 1935), Seitz EK (Wenckebach and Kunert, 1937, Rake and Shaffer, 1939 1940)

Cultivation

It has been reported that the virus can be grown in plasma (diluted with saline 1.6 to 1 7), and that sterile filtrates of such cultures provoke a measles-like reaction

Papp, 1936*a*, Phillips, 1938). The neutralizing power develops by the seventh day of the disease, but is much more potent by the thirteenth (Papp, 1936*b*). Debré suggested that the reaction might serve as an approximate guide to the potency of convalescent serum Papp (1936*a*), however, found that serum might be quite effective in sero-attenuation of measles but fail to give a positive Debré reaction. The reaction is of no value during the period of the eruption, as blanching does not occur (Kingsbury, 1928).

Virus neutralizing antibody can also be demonstrated by its power of inhibiting the development of lesions on the chorio-allantois (Mayer, 1941).

Orlov (1945) has reported that immune serum fixes complement with nasopharyngeal washings or a suspension of lungs of mice inoculated nasally.

Seroprophylaxis of Measles

A variety of biological agents may be employed in the prevention of measles, and it is convenient to use the term "seroprophylaxis" for this technique although the substances do not all, strictly speaking, consist of serum

1. Animal serum.

Certain sera prepared from animals by injection of cocci and other bacterial products have been used in prophylaxis The best known of these are Tunnicliff's measles antitoxin (goat or horse), Ferry and Fisher's measles antitoxin (horse), and Degkwitz's immune serum (sheep)

Tunnicliff's sera were reported on favorably by Tunnicliff and Hoyne (1926*a*, *b*), children exposed to infection did not contract the disease after prophylactic injection of these anticoccal goat and horse sera Halpern (1928) and Peterman (1930) also reported favorably Gunn (1928) found that only about 40 per cent. of contacts became protected after injection of Tunnicliff's serum as against 95 per cent. after convalescent serum Barenberg, Lewis, and Messer (1930) found the serum useless.

Ferry and Fisher's antitoxin was said to protect susceptibles (Ferry *et al.*, 1928, Ferry, 1929) Gunn (1928), however, found that no persons were protected after its injection

Another of these agents was Degkwitz's serum, which was supposed to have been prepared by injection of sheep with various measles exudates Ape serum was also used in some cases The serum was reported on favorably by Degkwitz (1926*a*, *b*, 1927–8), but others found it of little or no use (Zoepffel, 1927, Gunn, 1928, Leiner, 1929, Silverman, 1929, and see McCartney, 1927).

2. Human serum or plasma: convalescent and adult.

It was shown by Nicolle and Conseil in 1918 that convalescent measles serum had the important property of preventing infection in contacts if injected early in the incubation period Rietschel in 1921 showed that whole blood or adult serum was equally effective Debré and Ravina made the important discovery in 1923 that if serum was injected later in the incubation period a mild abortive attack of measles developed The serum can be either convalescent or adult and may be administered in various ways The term "serum" is usually employed although actually plasma is often used Whole blood may also be tried

Convalescent serum has been used by a large number of workers in many parts of the world, and is probably the most popular (e g, by Nicolle and Conseil, 1918, Kutter, 1921, Maggiore, 1921, Zschau, 1921, Blackfan, Peterson, and Conroy, 1923, Zingher, 1924, Copeman, 1926–7, 1929, Park and Freeman, 1926, Benson and Lawrie, 1927, Miller and Smith, 1927, Johannsen, 1928, Gunn, 1928, 1932*a*, *b*, 1938*a*, *b*, Nabarro and Signy, 1929, 1931*a*, *b*, Warwick, 1929, Barenberg, Lewis, and Messer, 1930, Pierret, 1931–2, Hale, 1934, Elkington, 1936, Hyland and Anderson, 1937*a*, *b*,

CHAPTER XXII

IMMUNITY IN MEASLES

EXPERIMENTAL ASPECTS

MONKEYS RECOVERED from infection following inoculation of human material or egg virus are resistant to a subsequent challenge (Blake and Trask, 1921 *c*, Rake and Shaffer, 1939, Hurst and Cooke, 1941; Rake, Shaffer, and Jones, 1941; Konova *et al*, 1945). Guinea-pigs also become resistant (Duval and D'Aunoy, 1922 *a*, Kawakubo, 1932, Marotta, 1939 *b*) Virus neutralizing antibodies have been detected in the *sera of guinea-pigs (Marotta, 1939 b)*.

IMMUNITY REACTIONS IN MAN

Hereditary Immunity

Butler (1912–13) found that from 0–6 months of age, 10 2 per cent. of contacts contracted the infection, while from *6–12 months* the *figure* rose to 77 per cent suggesting the presence of a degree of hereditary immunity (see also Stillerman, Marks, and Thalhimer, 1944).

Active Immunity Following Infection

After an attack, the acquired immunity is usually lifelong in character and of high degree For example, the Faroe islands were visited by measles in 1846, yet persons recovered from the last epidemic in 1781 were immune, despite a lapse of 65 years (Panum, 1847) Relapses are uncommon and occur within 1 month of the original attack (see Desbouis, 1919, Macciotta, 1925; Rolleston, 1926, and Stenn and Stenn, 1939) Second attacks have likewise only rarely been recorded (see Salzmann, 1920, Macciotta, 1925, Erdheim, 1926 *a*, Woolnough, 1929, Pelner, 1941) Matthews (1932) has recorded 4 attacks in 1 case Fouad (1938) recorded a number of second attacks in Egyptian children, 6 children suffered from 2 attacks in the same year

Stocks has emphasized the fact, on statistical evidence, that for every 1 declared case of measles, some 4 to 5 must acquire immunity through latent immunization (see above) Stillerman, Marks, and Thalhimer (1944) found that the percentage of contacts escaping infection increased steadily from ages 1–7 (10–14 per cent) to *10–15 (85 per cent)*

Following the injection of convalescent serum for sero-attenuation, *vide infra*, immunity is acquired

Active Immunity Following Vaccination

Taniguchi *et al*, (1935) produced localized eruptions by intradermal injection Herrman (see 1929) introduced a method of active immunization, which involved applying infective nasal secretion to the nasal mucosa A covering dose of immune serum was given shortly afterward Good results were claimed Latterly, living egg-cultured virus has been used in children, and is said to give some protection against natural infection (Maris *et al*, 1943, Rake, 1943)

Serum Antibodies

Debré and Ravina (1923) reported that if measles convalescent serum was *injected* intradermally in the prodromal period, no rash developed in that particular area This reaction was described independently by Keller and Moro (1925) Other workers have confirmed these findings (e g, Baar, 1931, Nabarro and Signy, 1931 *a*,

adult whole blood 20 c.c. For children over 3, give double these amounts. For attenuation, give the same doses on or after the 6th day, alternatively half the dose may be given within 5 days. A common practice in calculating the dosage of convalescent serum for children over 3, is to multiply the age by 2.

Serum retains its full potency, on ice, for 6 months, thereafter the titer falls to 60 per cent where it remains for a further 18 months (see Burnet, 1935).

Route of injection. Serum is usually injected subcutaneously or intramuscularly. It has been claimed that conjunctival instillation is effective (Anderson and Gérard, 1930) Blossom (1945) gave the serum intradermally (0.4 c.c.) on successive days, giving a total of 2 c.c.

Results It is generally agreed that in a very high percentage of cases measles can be absolutely prevented. Estimates of the percentage of cases which fail to be protected by serum injection given early in the incubation period vary from 2–5 to 20 per cent (Gunn, 1928, 1932 *a*, *b*, Pierret, 1931–2; Hobson, 1934)

Gunn (1938 *b*) has, however, expressed his disappointment with the results obtained by serum administration in the 1935–6 London epidemic; these results have also been fully described in an official *Report* (1938). A protection rate of 72.4 per cent. was obtained in the case of children inoculated with adult serum for prevention, in the 2 previous epidemics the results were 78.3 and 78.6 per cent, respectively. Convalescent serum gave a protection rate of 72.3 per cent. in contrast to 80.8 and 90.7 per cent previously. With regard to attenuation, the rate for adult serum was 31.5 per cent, compared to 38.5 and 37.4 per cent. previously, and for convalescent serum, 20 per cent. compared to 54.3 per cent. previously.

In earlier reports from various authors it appeared that attenuation is the rule if serum is given at the correct time, and it is probable that technical reasons may explain some reported failures. Thus, serum may have been stored for some period. Further, in any large scheme donors may not always be carefully selected and may yield a serum of low titer Another important factor which affects the degree of efficacy of prophylactics is the degree of intimacy of contact (Karelitz and Karelitz, *vide infra*). It is often difficult to judge accurately the day of exposure. The administration of serum may have the effect of prolonging the incubation period, even up to 4–5 weeks

About 3 to 4 per cent of children may show a general reaction, with slight rise of temperature, anorexia, and some local reaction after serum administration (Pierret, 1931–2).

Hepatitis may follow the inoculation of icterogenic batches (Medical Officers, Ministry of Health, 1943). It is hoped that ultraviolet irradiation may destroy any icterogenic agent present in serum.

Municipal schemes Many corporation public health departments have elaborated schemes for the collection, storage, and supply of suitable sera Adult cases of measles and older children are bled during convalescence, adult donors who contracted measles in youth may also be employed Boy Scouts and members of other youth organizations will usually volunteer for such service if asked The serum is generally prepared in a bacteriological laboratory where it may appropriately be examined for sterility and by the Wassermann test. The serum should be stored in a cool place The supply is usually limited and serum is only issued to local practitioners and hospitals for certain specified cases Such schemes have been described by various writers (see, e g, Copeman, 1926–7, Okell, 1932, Williams, 1936, Lemierre, 1937, Gunn, 1938 *b*, *Report*, 1938, Thalhimer, 1938).

Serum globulin The globulin of immune serum has been extracted and found to contain specific antibody, this extract is as powerful as the original serum and has been used prophylactically (Karelitz, 1933–4, 1938, Thalhimer, 1940). Gunn (1938 *b*) has found that both pseudo- and eu-globulins are effective. In the 1935–6 London epidemic the globulin fractions of adult serum gave a protection rate of

Lemierre, 1937, Hobson, 1938, Top and Badger, 1941; De Haas and Posthuma, 1942; Council on Pharmacy and Chemistry, A. M. A., 1943).

Adult serum may be used. Adult serum has been recommended by various authors (e g, Rietschel, 1921; Zingher, 1924; Siegel and Ermann, 1930, Barenberg, Lewis, and Messer, 1930, Morales and Mandry, 1930; Burn, 1931; Karelitz and Schick, 1932; Hunter, 1933, Brincker, 1936, Papp, 1936 c; Hyland and Anderson, 1937 b, Gunn, 1938 b, Champtaloup, 1939, Lempriere, 1939; Bull, 1940; Barenberg et al., 1942; MacKendrick and Seville, 1946).

Reconstituted plasma as used in transfusion work may be employed. Parental serum has been recommended by Barenberg *et al.* (1939).

Preparation of serum. As regards the time of collection, between 7 and 9 days after defervescence is regarded as optimal, and certainly not later than 12 to 14 days (Nabarro and Signy, 1931 *a*, *b*, Pierret, 1931–2).

The method of collection used by Park and Freeman (1926) involves drawing off the blood and running it into a 500 c.c. bottle containing 2 c c. of 25 per cent sodium citrate and 0.3 gm. of oxyquinolin sulfate. The red cells are allowed to settle by gravity in the refrigerator, or by centrifugation. The plasma is sealed into 3 c c and 6 c c ampules and 30 c.c. bottles. Wassermann and sterility tests are, of course, carried out on each sample A similar method was advocated by Nabarro and Signy (1931 *a*).

The method we have used involves the separation of serum from the patient's clotted blood, filtration of pooled samples is then carried out through a large Seitz filter. The serum is finally filled into ampules and stored until required. Wassermann and sterility tests are, of course, performed.

Another excellent method of preparation is to desiccate the serum *in vacuo* in ampules and then, before use, add sterile distilled water to reconstitute the original bulk.

Method of administration According to the day on which the serum is administered, so one hopes either entirely to prevent any attack developing, or, alternatively, to produce a mild modified illness. Serum administered in the first 5 to 6 days of the incubation period usually protects completely, while between the 6th and 9th days it results in a modified attack The day of appearance of the rash in the primary case is regarded as the 4th day.

After the 9th day the illness is seldom prevented or modified, although it has been claimed that the administration of 40–50 c c. of serum intravenously in the catarrhal stage has a beneficial effect (Kohn, Klein, and Schwarz, 1938, 1941; Thalhimer, 1940, Dungal, 1944).

Complete protection should be aimed at in certain circumstances Thus, the following types of case should be injected with serum early in the incubation period feeble children, those suffering from another disorder, children under 3, particularly during cold weather

The production of attenuated measles is the most satisfactory because children develop an active immunity Serum is usually given between the 6th and 9th days after exposure The modified attack is characterized by fever, slight catarrh, and a sparsely distributed rash The rash is papular and occurs on the face and rarely the limbs. Koplik's spots are not found and the illness only lasts for about 24 hours There is not much information available regarding the after-history of persons who have suffered from modified (sero-attenuated) measles, but Townsend (1936) recorded that no measles developed in 32 cases who had suffered from modified measles 10 years before

Dosage Varying schemes have been proposed for calculating the dosage (Report of L.C.C., 1938, Morales and Mandry, 1930, Burnet, 1935, Brincker, 1936, Stillerman, Marks, and Thalhimer, 1944). The Medical Research Council (1944) in Britain recommended the following doses for children under 3, for protection, given within 5 days of exposure convalescent serum 5 c c, adult serum 10 c.c.,

hicles, parties, and playgrounds In fact, the value of prophylactics should be estimated on the results of inoculating home contacts They found that the greater the hygienic state of the home the more efficient became the injection of prophylactics An especially important factor was found to be the possibility of isolating the case as early as feasible. In homes of poor hygiene, they found that the larger the dosage of prophylactic the higher the rate of complete protection

4. Gamma globulin.

In the future, as supplies become available, undoubtedly the prophylactic of choice will be the gamma globulin fraction of plasma prepared by Cohn *et al* (1944) Already a number of favorable supe-
riority (Greenberg, Frant, and Rurstein Jen-
nings, and Janeway, 1944, Stokes, Mar 945)
Janeway (1944) recommends the intramuscular injection on the 5th day after exposure of 0 1 c c to 0 075 c c per lb body weight for complete protection, 0 025–0 02 c c on the same day will attenuate

Reactions after the use of gamma globulin are rare, and it is probable that it can be heated sufficiently to destroy the icterogenic agent

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77 3 per cent. (*Report*, *1938*). It has been suggested that the potency of placental extract (*vide infra*) is dependent on its content of serum globulin

Whole blood. The use of whole blood (citrated), e g., from a parent, has been recommended by various workers in lieu of immune serum (Rietschel, 1921, Bader, 1929, Stewart, 1932, Lord, 1933, Culbert, 1938, Morley, 1945). As a dose some 10–20 c c should be injected intramuscularly The results seem to be as good as those obtained by other methods

3. Placental extracts.

It has been discovered in recent years that an extract prepared from human placentas has an action comparable to that of convalescent serum The introduction of this substance is largely due to the work of McKhann and his colleagues Thus McKhann and Chu (1933 *a*) reported that placental protein extracts contained antibodies to diphtheria toxin, to the erythrogenic streptococcal toxin, to poliomyelitis virus, and had a prophylactic effect in measles They recommended intramuscular injections of extract in the incubation period for protection or attenuation These observations have been confirmed repeatedly (McKhann and Coady, *1934*, McKhann, Green, and Coady, *1935*, Levitas, *1935*, Eley, *1935*, McKhann *et al*, 1935–6, Chu and Chou, 1935–6, 1936, Huber, 1936, McGavran, 1936, McKhann, 1937 *a*, *b*, Goldstein, Eisenoff, and Blauner, 1937, Rohr, 1938, Gunn, 1938 *b*, Huber and Kurz, 1938, Bundesen *et al*, 1940, Denhoff, 1940, Peragallo, 1940, Lyall and Murdick, 1941, Goldstein, 1942, Greenberg, Frant, and Rutstein, 1944).

Extraction. The active principle can be extracted from placentas by 2 per cent. sodium chloride and refined by precipitation with ammonium sulfate (McKhann and Chu, 1933 *b*) Methyl alcohol precipitation is also effective (Chu and Chou, 1935–6, 1936, Chu and Liu, 1944) Strean *et al.* (1941) prepared a globulin solution from blood from the placental end of the cord Thalhimer and Stillerman (1939) found protective substances in ascitic fluid and its globulin fraction

Titration In view of the fact that placental extract contains diphtheria antitoxin as well as measles antibody, it has been suggested that the extract be titrated by estimating its content of this antitoxin in units (Karelitz, Greenwald, and Klein, 1934–5)

The dosage of placental globulin for injection may be calculated by comparing its diphtheria antitoxin titer with that of the pooled placental blood, which has the same antitoxin titer as the mother's blood (Karelitz and Greenwald, 1934–5; Karelitz, Greenwald, and Klein, 1937 *a*, *b*) Thus, if the placental globulin gives twice as high an antitoxin reading as placental blood, then one would inject only half as much as would be the case with human serum

Rake (1939) titrated the antibody content against swine influenza virus

Administration The dose of dried protein extract should be 0 02 to 0 025 gm. per kilo body weight (Chu and Chou, 1936) The extract is usually administered intramuscularly, but it has been reported that absorption occurs after oral administration (Robinson and McKhann, 1935, McKhann *et al*, 1935–6)

Results Most observers have expressed their satisfaction with placental extract, and have regarded it as efficient as convalescent serum in prophylaxis or seroattenuation (see *J Amer med Ass*, 1938) Some observations of McKhann (1937 *b*) show, however, that the immunity conferred is relatively short-lived, apparently not much over 2 to 3 weeks (see also Parish, 1938), Gallagher (1942) also had disappointing results Karelitz and Karelitz (1938) drew attention to an important factor that largely controls the efficacy of placental extracts, or other prophylactic substances That is to say, the type of exposure to infection must be considered, for they found that infection disseminated in the following sources was so irregular that no true estimate of the value of prophylactics injected into contacts could reasonably be made hospital and institution wards, nurseries, schools, public ve-

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rise of temperature 48 hours after defervescence, with a return of constitutional symptoms

The rash may be accompanied or followed by a hemorrhagic diathesis and purpura (Strom, 1940, Fox and Walton, 1946, Magnusson, 1946).

CONGENITAL LESIONS DUE TO RUBELLA

It has been reported only of recent years that babies born to women who contract rubella in the early months of pregnancy commonly show congenital defects

The first observations on this point were made by Gregg in Australia in 1941 (Gregg, 1942). Since then, numerous other cases have been reported from Australia, America, and elsewhere (Swan *et al*, 1943, 1944, Erickson, 1944; Evans, 1944, 1947, Reese, 1944; Rones, 1944; Swan, 1944; Albaugh, 1945, Altmann and Dingmann, 1945, Carruthers, 1945, deRoeth and Greene, 1945, Greenthal, 1945; Gregg, 1945, Krause, 1945; Long and Danielson, 1945; Perera, 1945, Fox and Bortin, 1946, Prendergast, 1946, Winterbotham, 1946) The defects that have been noted are cataract, micro-ophthalmia, uveitis, dacryostenosis, glaucoma, retinal pigmentation, strabismus, deaf-mutism due to loss of cochlear function, mainly from lack of development of the organ of Corti, cardiac lesions such as septal defects and patent ductus arteriosus, microcephaly, cerebral agenesis, and mental retardation, dental abnormalities such as retarded eruption, enamel hypoplasia, and caries, talipes valgus, spina bifida, and cleft palate, general physical retardation, obliteration of bile ducts, cryptorchism, hypospadias, and inguinal hernia, anemia and purpura

Infection appears to be most dangerous in the first 2 months of pregnancy, and probably nearly every baby will show some defect Infection in the third month will produce lesions in about half the babies. Probably attacks of rubella in the latter part of pregnancy do not cause congenital lesions. It does not appear that severe attacks are more dangerous than milder ones.

Swan (1944) studied the pathological features of some cases, and described patent ductus arteriosus, patent foramen ovale, and deficient interventricular septum The lenses showed necrosis of the nuclear portion The lungs showed bronchopneumonia Foci of degeneration were found in the liver. Fibrosis of the glomerular capillaries was also observed

Apparently, congenital defects were not attributed to rubella before about 1939, and it seems as if the virus may have undergone a mutation to a more invasive type in recent years It also seems as if these lesions are much commoner after rubella than after other exanthemata.

Findings of the Australian Committee A special committee examined this question, and reported substantially as follows (Director General of Health, Commonwealth Dept of Health, 1945)

1. That cases of congenital defects following maternal rubella during pregnancy occurred previous to 1940, but the relationship between maternal infection and the congenital defect was not recognized,

2. That it is impossible to estimate the number of pregnant women and subsequent children affected as a result of the epidemic of 1940,

3. That apparently children are not affected when the maternal infection occurs after the 4th month,

4 That the defects have been deaf-mutism, eye and heart disease, and possibly mental defect, and that the first 3 defects may occur singly or in combination,

5. That there is no relationship between the severity of the maternal infection and the nature of the defect

CHAPTER XXIII

GERMAN MEASLES (RUBELLA)

CLINICAL FEATURES

THE ILLNESS may be *initiated by a prodromal period a day or so before the appearance* of the rash, and is characterized by cough, sore throat, and enlargement of the glands in the neck.

The rash usually appears first on the face, but rapidly spreads. The constituent macules are smaller and less elevated than those of measles The eruption seldom lasts more than 36 hours Some catarrh usually persists during the eruptive phase, and the temperature is slightly raised. One of the most characteristic features is the presence of enlarged cervical, and often axillary, lymph nodes.

Another characteristic feature is the appearance of an absolute lymphocytosis Hynes (1940) carried out a special study of the blood picture, and made the fol- [illegible] his patients had an absolute neutropenia which [illegible] after the 10th day, a third showed an absolute [illegible] lymphopenia at onset changed to an absolute lymphocytosis after the 5th day. He usually found Turck cells to be numerous, they reached their maximum about the 4th day Plasma cells were present in up to half his patients in the first week. The leukocyte count can thus distinguish between rubella and scarlet fever, but not between rubella and measles. The differential diagnosis between rubella and glandular fever may be difficult and depend on the sheep cell agglutination test (Deumié, Brumpt, and Thomas, 1940–41).

COMPLICATIONS

Nervous Complications

Between 50 and 100 cases of severe nervous complication have been described The features are those of meningo-encephalitis, which develops between 1 and 6 days after the rash, usually about the 5th day of illness (Owen and Greenaway, 1940, Bradford, 1943, Falger, 1944, Wingo, 1945). Margolis, Wilson, and Top (1943) described 14 cases, and reviewed 34 from the literature They gave the following description the earliest complaints are headache, vomiting, stiffness, and pain in neck and extremities The reflexes are absent, unequal, or exaggerated Speech defects and muscular twitchings may occur.

In severe cases there are convulsions and coma. The course of the illness is usually short In their series, 4/14 died within 3 days of onset, and in previous cases 6/34 died

The CSF shows a marked increase of protein, with less marked increase of lymphocytes Margolis, Wilson, and Top found that the average count was around 90 cells per c.mm.

Only a few reports on the pathological features have appeared Margolis, Wilson, and Top found edema of the brain, there was perivascular infiltration with plasma cells, lymphocytes, or mononuclears Demyelination was not found, but there was nerve cell damage Capillary hemorrhages were found in the floor of the 4th ventricle.

Myelitis may follow rubella (Morris and Robbins, 1943). Neuritis has been recorded (Harrison, 1940, Hodges, 1940, Sprott, 1940).

Other Complications

Rheumatic complications have been described (Murray, 1941) Bennett and Copeman (1940) studied a number of cases in soldiers. There were transient joint

ably develop in convalescence Pooled plasma has been used as a prophylactic, and Barenberg *et al* (1942) claim good results

A proportion of monkeys convalescent from experimental infection proved immune to reinoculation (Habel, 1942).

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6 That if the maternal infection occurs after the 2nd month of pregnancy, there is less likelihood of eye disease;

7. That a large number of the affected children are below normal birth weight,

8. That the majority of the children are below average weight for age, and that they show a degree of microcephaly, and their general physique is below standard;

9. That many of the children are late in sitting up and walking, and difficulties in feeding are common,

10. That during the first few years of life, signs of general instability of the nervous system of the children are common,

11. That the deafness is not absolute, and the apparent improvement in hearing about the 2nd half of the 4th year is secondary to improvement in the power of concentration;

12 That the main dental abnormality is retardation of eruption, but that at 3½ years all the children had a full complement of teeth;

13 That very few of the children are mentally defective though many are mentally retarded, the majority of the children are educable, and will be fit to earn their own living, following appropriate education,

14 That there is no evidence to show that the occurrence of rubella during pregnancy has any harmful effects on the mother.

DISTRIBUTION OF THE VIRUS

Habel (1942), by monkey inoculation, found virus in the blood up to 30 hours after the appearance of the rash, nasal washings were positive up to 24 hours He also isolated virus from these sources in a case without a rash.

EPIDEMIOLOGY

The incubation period varies from 5–21 days, with 14 days as the average.

Although predominantly a disease of childhood, adults are attacked more commonly than occurs with most other exanthemata Outbreaks among students and service personnel are not unusual (Wildman and Teasey, 1940)

There is usually a seasonal prevalence in spring and early summer.

The disease is presumably spread by droplets, as virus has been recovered from the nose The case is probably infective for only a few days, from shortly before until shortly after the appearance of the rash Smith (1947) has found it safe to allow return to school after only 4–5 days away

PROPERTIES OF THE VIRUS

The infection has been transmitted to children by inoculation of filtered nasal washings subcutaneously (Hiro and Tasaka, 1938).

The rhesus monkey (*Macacca mulatta*) has been found to respond to the inoculation of human blood or nasal washings by developing a mild illness (Habel, 1942). There is a leukopenia after an incubation period of 8 days. Fever and lymphocytosis follow 2 days later Then there develops a macular rash on the face, chest, abdomen, and thighs Animals can be infected by the nasal, subcutaneous, intraperitoneal, or intravenous routes Virus can be found in the monkey's blood at the time of the rash, and in the period of leukopenia and fever in those animals that do not develop a rash

Habel (1942) was able to pass the virus through the chorio-allantois, no obvious lesions were produced, but membrane suspensions were pathogenic to monkeys He found no evidence of cross immunity with measles virus

IMMUNITY

Second attacks may occur, but are not common It is probable that immunity may be acquired following subclinical attack Virus neutralizing antibodies prob-

sions (cytoplasmic and nuclear) have been described in epithelial cells. (Sohier and Jaulmes, 1939, Sohier and Nabonne, 1939; Sohier and Levrat, 1945).

Cytological examination of the saliva shows a few leukocytes and epithelial squames from Stensen's duct. There is no abnormality in the fermentative power of the saliva (De la Prade and Loiret, 1919 *b*). The calcium and chloride contents increase for the first fortnight or so (Rocchi, 1929, 1930).

The femoral glands may be involved (Schmidt, 1945).

Parotitis, and more especially involvement of the submaxillary glands, may be complicated by edema of the tissues of the anterior wall of the thorax and sometimes abdomen (Barker, 1943; Rappaport, 1943, Gellis and Peters, 1944; Chrysanthis, 1946).

2. Orchitis.

This is probably the second commonest manifestation of mumps, or alternatively, the commonest complication of parotitis. Numerous authors have described the condition and only comparatively few can be mentioned by name (Hamilton, 1790; Dukes, 1900; Smith, 1912; Feiling, 1914–15; Capitan, 1918; Radin, 1918; Dumitresco, Jonnesco, and Chiser, 1932; Manca, 1932; Bieberbach and Vibber, 1933; Huber and Lièvre, 1935; Montgomery, 1936, Ohlmacher, 1936; Zax, 1937). In addition, the subject has been reviewed by Wesselhoeft (1920) and more recently by Stengel (1936). The following account is largely based on that of Stengel. The virus probably reaches the testis from the blood, although it was at one time suggested that the infection was carried to the urethra by infected hands (Sailer, 1919–20). Most agree that the body of the testis is mainly involved (i.e., orchitis) with a degree of accompanying epididymitis. Feiling (1914–15) holds, however, that epididymitis is primary.

Predisposing causes. The fact that the testicle is constantly exposed to minor traumata has been held to account for the localization of the virus therein. It is doubtful whether this factor plays a part, because although Dukes (1900) treated 30 boys by absolute confinement to bed for 8 days, 20 per cent. developed orchitis. Dukes's observation has been confirmed by many others. It seems to be established, however, that undue sexual activity is a definite predisposing cause (see Bardachzi and Barabas, 1920).

Incidence. From a review of 1,000 cases of parotitis Stengel concluded that approximately 18 per cent. are complicated by orchitis. The disease is predominantly one of puberty and adolescence; young soldiers appear to be frequently attacked by this complication. During the first and second World Wars, mumps, and especially orchitis, was a serious cause of loss of manpower. A number of authors alluded to this question in the second World War (Gordon and Heeren, 1940, Wesselhoeft and Walcott, 1942, Dermon and Le Hew, 1944, McGuinness and Gall, 1944, Potter and Bronstein, 1944; Candel, Wheelock, and Grimaldi, 1945).

Time of onset. Orchitis usually develops about 4 to 7 days after the onset of the parotid swelling. A number of well-authenticated cases of primary orchitis have, however, been recorded (e.g., by Bieberbach and Vibber, 1933, Montgomery, 1936, Ohlmacher, 1936, Zax, 1937, 1943; Hild, 1938, Coe, 1945; and see Stengel, 1936) In such cases help can be derived from the CFT (see below).

Course The disease occurs 2 to 3 times more commonly unilaterally than bilaterally, and if the other testicle does become affected it usually does so a day or so after the first (Stengel) The temperature often rises with the onset of orchitis and there is a general febrile reaction. The organ rapidly swells to several times its normal size and is acutely tender and painful; there may be a slight urethral discharge. The pain may be so severe as not to be relieved even by morphia. In some cases operation with incision of the tunica has been performed. The blood usually shows a lymphocytosis. The affected testis frequently atrophies and this may be due to an acute hydrocele strangulating the testis (Burhans, 1945). Sterility has

CHAPTER XXIV

MUMPS (EPIDEMIC PAROTITIS)

AN ACUTE infectious disease *characterized by swelling of the parotid glands has* been known since the days of Hippocrates (see Gordon, 1914, Hansen, 1934). In later times Hamilton (1790) made a special study of the condition in Norfolk (England) and clearly showed that mumps was more than a simple condition causing parotitis, as it might involve the testicles as well. French workers have stressed the frequency of nervous manifestations.

Earlier attempts to prove that the causal agent was a cultivable organism were unconvincing. That the etiological agent was in fact a filtrable virus was suggested by Granata (1908 *a, b,* and see Kolle and Wassermann, 1913), but Gordon (1914) was the first to show clearly that the disease could be transmitted to animals, thus laying the foundation for later work. Wollstein (1916, 1918, 1921), Findlay and Clarke (1934), Johnson and Goodpasture (1934, 1935, 1936 *a, b*), and others (*vide infra*) have proved that the causal agent is filtrable and transmissible to animals

A useful review of the literature on mumps has been published by Rolleston (1932).

CLINICAL FEATURES

The term "mumps" is frequently regarded as synonymous with "epidemic parotitis," but this is somewhat inaccurate. Epidemic parotitis is certainly the commonest manifestation of infection by the virus of mumps, but it is not the only one. The other manifestations of mumps are orchitis, meningo-encephalitis, pancreatitis, and ovaritis, these most commonly follow the development of a parotitis by some 5 to 7 days and in such cases may be regarded as complications of epidemic parotitis In other cases, however, these manifestations may either precede parotitis or occur independently.

We prefer, therefore, to use the term "mumps" to include the various manifestations mentioned, and to restrict the term "epidemic parotitis" purely to involvement of the parotid glands

Manifestations of Mumps

1. Parotitis, and inflammation of the submandibular glands.

Following an incubation period of 18 to 21 days there may be a prodromal period of 12 to 36 hours in which headache, posterior pharyngitis, and general nonspecific pyrexial symptoms occur. The onset of the disease is characteristically sudden, with pain in one or both parotid glands. Sometimes the submaxillary and sublingual glands may be involved, with or without affection of the parotids (Smith, 1943) The parotid rapidly swells, the hollow below the ear becomes filled up, but the gland is not usually tender, and the overlying skin is not discolored. There is very considerable pain on attempting to open the mouth, which is usually very dry The orifice of Stensen's duct is often bright red.

General pyrexial disturbances are noted for a few days, and headache may be severe. The spleen may be enlarged (Greene and Heeren, 1937) The majority of patients feels acutely uncomfortable for a few days, but the swelling and accompanying malaise usually disappear in 7 to 10 days In bilateral cases both glands may be involved *de novo*, but more commonly the second gland becomes swollen a few days after the first.

Specimens may be taken with a suitable aspirator direct from the duct, inclu-

in which the virus of lymphocytic choriomeningitis cannot be found, are due to mumps (Howard, 1919, Paddock, 1932, Armand-Delille *et al.*, 1937, Jersild, 1942, Applebaum, Shrager, and Paff, 1945, Coe, 1945; J. H Smith, 1945). In such cases the diagnosis may be confirmed by the CFT on blood or cerebrospinal fluid (Kane and Enders, 1945, Henle and McDougall, 1947).

Symptoms The commonest symptoms are referable to an inflammatory meningeal reaction, headache being usually very severe; the patient is anxious, excited, restless, and even delirious Convulsions and contractions may be noted, the temperature is usually raised Many authors have noted evidence of severer parenchymatous damage due to an encephalitis Thus the following symptoms have been recorded mania and mental confusion (Urechia and Elekes, 1936), cerebral diplegia, dementia, and choreo-athetosis (Stroé and Chiser, 1932), aphasia, paralyses, and hemiplegia (Monro and Healy, 1883, Lannois and Lemoine, 1886), symptoms of acute midbrain disease (van Bogaert and Meunier, 1938). The diagnosis of encephalitis lethargica may easily be made until the appearance of parotitis gives the clew (Bedingfield, 1927). The cases in which meningitis is the main feature are often followed by orchitis

Gordon (1914) [illegible] which most of the children died [illegible] time of onset The patients were [illegible] and eventually became comatose. [illegible] pupils were dilated, and there were muscular movements of twitchings or convulsions Lymphocytosis occurred in the blood and cerebrospinal fluid Pathologically the central nervous system was remarkably little involved, although it showed some congestion and early nerve cell degenerative changes The salivary glands showed an acute interstitial inflammatory reaction.

4. Pancreatitis.

This manifestation is comparatively rare, nevertheless a number of cases have been recorded (Sabrazès, Broustet, and Beaudiment, 1927, Spicer, 1933, James, 1933, Greene and Heeren, 1937, Herson, Christopoulos, and Coghill, 1944). Farnam (1922) and Brahdy and Schaeffer (1931) have reviewed this subject, and the following account is largely based on their works.

The disease usually develops some 5 to 11 days after the onset of parotitis, although rarely the pancreatitis may precede In fact, it was suggested in 2 cases that mumps pancreatitis occurred without any parotitis (Friedjung, 1928) The patient is febrile (up to 103° to 104° F.), nauseated, and complains of headache, abdominal pain, and tenderness Vomiting is not a prominent feature, the bowels are constipated and there is no steatorrhea In very severe cases the urine may contain sugar, as well as giving a high reading in the diastase test Although very rare, a few cases of diabetes following mumps pancreatitis have been recorded (see Patrick, 1924, Couronne, 1927, Rennie, 1935). These cases are often of severe type and terminate fatally Apart from the development of diabetes, mumps pancreatitis seldom proves fatal

5. Ovaritis.

This manifestation is the rarest of all and in no way corresponds in frequency to the parallel condition of orchitis.

The manifestation of ovaritis was [illegible]

[illegible] between lower abd[illegible] [illegible] the infection is associated with a high temperature and [illegible] ingitis may readily be made app[illegible] onset of parotitis, but may has [illegible] 1936) Suppurative ovaritis [illegible] 1934)

been held to be unusual owing to the rarity of bilateral involvement (see Capitan, 1918, Bénard, 1928), but Seguy (1942) estimates that 4 per cent. of cases of sterility are due to mumps. There is probably no direct relationship between orchitis and tumor growth (Gilbert, 1944).

3. Meningo-encephalitis.

There is probably a lymphocytosis in the CSF in most cases of mumps, whether there is clinical evidence of nervous involvement or not, the term "latent meningitis" has been used to describe such cases, where the diagnosis can only be made by examining the fluid pathologically, counts up to 2,500 per c mm. have been found in declared or [illegible] protein is also increased (Monod, 1902; Chauffard [illegible] 4, 1905; Feiling, 1913, Silwer, 1936, Finkelstein, [illegible] oyer, 1938, Schonfeld, 1939, Bang and Bang, 194 [illegible] 1946). Sometimes the fluid may be virtually normal in declared cases of nervous involvement (Halcrow and Wang, 1945).

Various theories have been advanced to account for the development of meningo-encephalitis (see Montgomery, 1934):

(*a*) The cerebral condition is merely a passive congestion from compression of the jugular vein. This theory can attract little attention as it appears to be a quite inadequate explanation of the observed facts

(*b*) The condition is due to the lodgment of infected emboli from an endocarditis, but no such condition has been found post mortem

(*c*) It has been suggested that mumps is primarily a disease of the central nervous system caused by a neurotropic virus (Philibert, 1932, Dutrey, 1936) The virus [illegible] tis We feel [illegible] al evidence. [illegible] of a number of sites, such as the meninges, the parotids, the pancreas, the testes, or the ovaries This view may be correct, but it is difficult to understand why a blood-spread should be invoked to explain the common primary parotitis, for it is well known from both animal and human experiments that infection occurs regularly after application of virus to Stensen's duct (*vide infra*)

We think that the central nervous system is most likely to be involved by spread along axonal paths, in cases of primary meningo-encephalitis probably along the olfactory nerves or nerves from the nose or pharynx; in secondary cases perhaps along the facial and other nerves in relation to the salivary glands While the term "meningo-encephalitis" is usually applied in a general way, in some cases the symptoms are predominantly meningitic, and in others encephalitic

Attention was first drawn to the occasional involvement of the central nervous system in mumps by Gallavardin (1898). Since that time, numbers of authors have described cases (Monro and Healy, 1883, Lannois and Lemoine, 1886, Chauffard and Boidin, 1904, Dopter, 1904, 1905, Feiling, 1913, Bedingfield, 1927, Holtz, 1931, Paddock, 1932, Dumitresco, Jonnesco, and Chiser, 1932, Stroé and Chiser, 1932, Kousmine, 1934, Andrieu and Passa, 1935, Urechia, 1936, Silwer, 1936, Auvray, [illegible] Smith 1937 Finkelstein, 1938, Fuhrmann, 1938, Glanzmann, [illegible] 1939, 1940, Stewart and Edwards, 1939, Tabor [illegible] 41, Ahlberg, 1942, Levison and Thordarson, 1942, Carleton, 1943, Feer, 1943, Candel *et al*, 1944 *b*, Herson, Christopoulos, and Coghill, 1944, McGuinness and Gall, 1944, Steinberg, 1944)

Time of onset. The symptoms usually develop from 4 to 7 days after the onset of the parotitis, when this swelling is beginning to subside Cases have been recorded where meningo-encephalitis preceded the parotitis or orchitis by a few days (Bedingfield, 1927, Urechia, 1936, Urechia and Elekes, 1936, Auvray, 1937, Harris and Bethel, 1938, Snapper, 1940) Some cases of aseptic meningitis without any parotitis,

Myocarditis has been described (Wendkos and Noll, 1944, Rosenberg, 1945 *a*, *b*, Felknor and Pullen, 1946).

A case of pituitary basophilism following parotitis with orchitis has been recorded (Huber and Lièvre, 1935). Another case in which pituitary affection was probably present was recorded by Lesné, Dreyfus-Sée, and Lièvre (1930).

Although mumps very rarely occurs during pregnancy, the outlook is serious. Thus Dutta's (1935) case died from acute nephritis, Moore's (1931) patient was delivered of a macerated fetus, while another case died from nephritis and eclampsia (Micheleau, 1927).

CLINICAL PATHOLOGY

The blood. Various authors have examined the blood in mumps, and the following facts seem to be fairly well accepted (Feiling, 1913, 1914–15; Jahn, 1928, Matsumoto, 1936; Montgomery, 1936): (1) There is a relative or absolute lymphocytosis, counts ranging up to about 15,000 cells per c mm, of which 45 per cent. may be lymphocytes (2) This lymphocytosis may be found from the 1st to the 14th days of the illness (3) The examination of the blood may be of value in the differential diagnosis from secondary (septic) parotitis.

The blood sedimentation rate is raised in orchitis (Candel *et al.*, 1944 *a*). The blood diastase is usually increased (Faergeman, 1943, Murphy, Bozalis, and Bieri,
1 lebaum, 1944, Fennel, 1944;
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lymphocytosis, this becomes still more definite in cases of meningo-encephalitis (see above).

DISTRIBUTION OF THE VIRUS IN INFECTED PERSONS

It is generally agreed that virus can only be isolated from the saliva of cases of parotitis for 2 to 4 days after onset. On epidemiological grounds, however, it is probable that the virus actually appears in the saliva 3 to 4 days before the onset of clinically apparent parotitis The virus has been found in the cerebrospinal fluid of a case of meningitis by Lavergne *et al.* (1937 *a*, *b*), and Swan and Mawson (1943), as well as Henle and McDougall (1947), also in the blood of a severe case by Wollstein (1918).

MORTALITY

The mortality from mumps is remarkably low, the majority of deaths occurring under 5 years and especially under 1 year of age. The average death rate is probably about 3 per million living. The majority of deaths are due to meningo-encephalitis, pancreatitis, or a superimposed complication such as nephritis

PATHOGENESIS

Parotitis can be reproduced in monkeys by injecting the parotid ducts, and in many cases by directing a spray of infective material at the duct orifices (*vide infra*) This experimental work has made it almost certain that, in man, epidemic parotitis follows the introduction of infected material into the mouth. Having reached the parotid and caused infection, the virus may enter the blood and localize in the meninges, testes, pancreas, or ovaries. Virus may reach the central nervous

COMPLICATIONS

Nervous Complications

Various lesions of the nervous system may occur which are to be regarded as true complications rather than as evidence of a primary localization of the virus (see Glanzmann, 1938).

Myelitis. Symptoms referable to the cord are rare, but a definite case of transverse myelitis was recorded by McKaig and Woltman (1933). Fortney (1937), Lemierre and Morin (1938), Lancaster (1938), Dénéchau (1939), McGuinness and Gall (1944); and Lightwood (1946) have also reported cases.

Ocular complications. Optic neuritis may occur on recovery from mumps (see Swab, 1938). A case of Young's (1933) became completely blind, but recovered full vision later. A temporary recoverable amblyopia may occur during convalescence (Hatry, see Gordon, 1914).

Other ocular nerves may also be paralyzed and some cases of Butler and Wilson (1937) illustrated such involvement. These ocular paralyses are probably due to a meningoradiculitis and not to a more central effect

Bonnet (1938) claimed that eye changes in mumps should be considered, not as complications, but as true manifestations, in the same way as are orchitis and meningitis. Among the lesions that he refers to are conjunctivitis, dacryocystitis, iritis, and keratitis.

Auditory complications. As long ago as 1898 Gallavardin collected some 50 cases of deafness due to mumps, and noted the presence of Ménière's syndrome in some of these. Boot (1908) also reviewed a somewhat similar number of cases, and found that this deafness was due to involvement of the cochlea, or the semicircular canals, or more commonly of both. Hubbard (1915) estimated that up to 5 per cent. of deaf-mutism in the United States was caused by mumps.

Herpes zoster. The presence of meningoradiculitis would probably account for the occasional record of zoster eruptions developing along the distribution of various nerves, e.g, the trigeminal (Sicard, 1905).

Peripheral neuritis. Paralysis from polyneuritis is rare, and may be due to a meningoradiculitis at the site of exit of the roots from the spinal column (Gallavardin, 1898, Thiriet, 1928–9, Collens and Rabinowitz, 1928, Church, 1946) Facial paralysis has been recorded (Dopter, 1904, Nemlicher, Tchernikow, and Solomonowa, 1932), this suggests that the virus may enter the nerve in the neighborhood of the parotid gland and spread centripetally. Harris (see Harris and Bethell, 1938) saw a case of paralysis of the serratus magnus due to involvement of the long thoracic nerve

Appendicitis

The onset of a serious, often gangrenous, acute appendicitis, about a week after parotitis, has been recorded on a few occasions (Sandler and Finne, 1932, Donnelly and Oldham, 1933, Finch, 1933, Leenhardt and Barnay, 1937) The obvious importance of such cases is that operation may be delayed in the mistaken impression that one is dealing with a mumps pancreatitis

Other Complications

A definite acute nephritis with hematuria, casts, and albumin may occur and contribute to a fatal issue (Feiling, 1914–15, Sabrazès, Broustet, and Beaudiment, 1927; Dutta, 1935).

Local parotid suppuration is a rare sequel (see Rundle, 1929). Upper respiratory tract infection may occur (Herson, Christopoulos, and Coghill, 1944) Keratitis has been reported (Danielson and Long, 1941)

It has been suggested that endocarditis may complicate mumps (Sciaux, 1935)

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The cerebrospinal fluid. Even in uncomplicated cases of mumps there is a lymphocytosis, this becomes still more definite in cases of meningo-encephalitis (see above).

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system by centripetal spread along nerves associated with the parotid gland, and it should be recalled that facial paralysis has been described.

It is more difficult to explain those cases in which meningitis, or other manifestation, precedes the parotitis or occurs as the sole evidence of mumps infection Here it is necessary to postulate that the virus enters the body by some route other than the parotid gland. In the case of primary meningo-encephalitis it is not unreasonable to assume that the virus may spread by the olfactory, nasal, or pharyngeal nerves. In the other cases the virus may enter the tonsil and be disseminated to the pancreas, testes, or ovaries by the blood stream.

Mumps virus resembles NDV and influenza viruses in agglutinating red cells (see below). Burnet (1945) therefore suggests mumps virus, like these agents, multiplies in superficial and epithelial cells of the respiratory tract or conjunctiva, and causes initially a subclinical infection This primary invasion produces enough virus to enter the blood and localize in the parotids or other organs

PATHOLOGICAL FEATURES

Remarkably few reports of the changes in the parotids are available for study (*Dopter and Repaci, 1909, Henke and Lubarsch, 1929*). The changes are predominantly interstitial with slight lesions of the excretory ducts. The connective tissue shows a serofibrinous exudate and is infiltrated with leukocytes, mainly perivascularly. The epithelial cells of the ducts show some degenerative changes, and the lumina are filled with necrotic debris and polymorphs Areas of hemorrhage are present

The testis was also examined by Dopter and Repaci as well as by Smith (1912) and Manca (1932) It is greatly enlarged and congested and shows punctate hemorrhages Histologically, there is an irregular involvement of the seminiferous tubules, some of which show degeneration of epithelium and a polymorphic infiltration By contrast, the interstitium is more uniformly affected and shows serofibrinous exudation and edema Numerous leukocytes are found, especially perivascularly. Gall (1947) traced a graded series of lesions from edema and perivascular lymphocytic exudate to a diffuse lymphocytic infiltration of the interstice with focal hemorrhage and destruction of germinal epithelium, with plugging of tubules by epithelial debris, fibrin, and polymorphs He found that in the epididymis changes were confined to the connective tissues

The pancreas has been examined by various workers (Lemoine and Lapasset, 1905, Farnam, 1922, Sabrazès, Broustet, and Beaudiment, 1927) The organ is enlarged, congested, and may show some fat necrosis

Changes in the central nervous system have been described by Maximowitch (1880) and Bien (1913) The brain appears congested and the surface is covered by a serofibrinous exudate, while there may be some destruction of the sheaths of the white fibers in the cortex Donohue (1941) found perivascular demyelination, as in other postinfective encephalitides

EPIDEMIOLOGY

Mumps occurs in all parts of the world In "virgin soil" the infection tends to be severe (Herson, Christopoulos, and Coghill, 1944) The majority of cases occurs either in childhood or adolescence A case has been recorded at the age of 80 (Paraf and Orestein, 1940) The disease is very prevalent among young soldiers and in residential schools (Medical Research Council, 1938) It appears that the disease does not tend to spread widely throughout a school The epidemic is usually drawn out, however, and occupies the greater part of a term.

In Norway it has been shown that mumps epidemics occur regularly every 8 to 10 years (Gundersen, 1927) In Britain, at any rate, the disease occurs more commonly in the autumn and spring than at other times Mumps appears to obey the special "epidemic rules" of Petersen (Heiberg and Petersen, 1946)

The disease is almost certainly spread by infective droplets of secretion, the virus is present in the saliva, from probably a few days before the onset of parotitis until a few days after The "striking-distance" of mumps is not very considerable, and it is unlikely that the virus has any great resistance to sunlight or desiccation The dissemination of the infection is chiefly due to declared cases, but sufferers from subclinical infections may play some part

As respiratory symptoms are uncommon, it is unlikely that mumps can be regarded as a true air-borne disease (Habel, 1945 *a*). However, Wells (1945) claims that irradiation of class rooms has some effect on incidence

A case of mumps should probably be isolated for 3 weeks In view of the long incubation period, contacts may be quarantined for 1 month It is probably unnecessary to enforce quarantine for the first week following exposure as cases do not usually become infective until at the most 3 to 4 days before the onset of parotitis The regulations of the British Board of Education, as expressed in their *Memorandum* of 1942, require the patient to be excluded from school for 1 week after the subsidence of the swelling Contacts need not be excluded Smith (1947) discusses the procedures to be followed in boarding schools

INVESTIGATION OF THE ETIOLOGICAL AGENT OF MUMPS

1 *Cultivable cocci* In the early days a number of workers reported the isolation of cultivable cocci from the parotid gland or testis (Laveran and Catrin, 1893, Teissier and Esmein, 1906, Korentschewsky, 1907, and see Gordon, 1914, and Kermorgant, 1925 *a*) In view of subsequent work no significance need be attached to these results, the organisms were either commensals or contaminants Those workers who have isolated the virus of mumps have failed to find any organisms in the parotid fluid (Nicolle and Conseil, 1913 *a*, *b*, Johnson and Goodpasture, 1934)

2 *Kermorgant's spirochete* Kermorgant claims to have isolated a spirochete from human lesions which can produce parotitis experimentally (Kermorgant, 1925 *a*, *b*, *c*, 1933) Kermorgant obtained the buccopharyngeal secretion from early cases of mumps and made cultures in anaerobic media The primary cultures were, of course, mixed, one of the organisms being a spirochete On repeated subculture it was eventually possible to obtain the spirochete and a motile bacterium growing together in symbiosis without any other organisms The spirochete was of the refringens type, and could be agglutinated and lysed by mumps convalescent sera The spirochete and bacterium together caused a parotitis in monkeys after injection into Stensen's duct When such cultures were filtered (Chamberland L_2 or L_3) the bacterium did not pass, but the spirochete passed through in a granular or filtrable phase The filtrate reproduced the same lesions as the original culture, including orchitis on intratesticular injection of monkeys On adding some of the motile organism to the filtrate and then culturing, the spirochete reappeared It was also claimed that orchitis developed after intratesticular injection of rabbits

Kermorgant himself was unable to demonstrate the spirochete in the infected

1927), nor in parotid secretions (Johnson and Goodpasture, 1934, Findlay and Clarke, 1934) Further, the spirochaeticidal arsenicals were ineffective in treatment (Molinelli, 1925) Kermorgant's results are not generally accepted, for, although he isolated a spirochete, it is unlikely that it was more than an unimportant commensal of the human mouth

3 *The filtrable virus of mumps* It is now generally agreed that the causal agent of mumps is a filtrable virus Granata was the first to suggest this possibility (1908 *a*, *b*, and see Kolle and Wassermann, 1913) He injected rabbits by various

routes with filtered saliva from human cases, and found that a definite rise of temperature occurred. After intraparotid injection a local swelling developed.

The next advance was made simultaneously by Nicolle and Conseil (1913 *a*, *b*) in France, and Gordon (1914) in England. Nicolle and Conseil injected fluid obtained by puncturing human parotid glands into the parotids of bonnet monkeys. Fever usually developed after the 27th day and in 1 out of 3 monkeys a definite painful swelling of the parotids was noted, the blood showed a lymphocytosis during the febrile period.

Gordon injected rhesus and cynomolgus monkeys intracerebrally with Berkefeld filtrates of human saliva obtained in the first few days of parotitis Some of the animals died on the fourth day with meningeal symptoms Following combined intravenous and intraperitoneal injection, a rhesus monkey developed swollen parotids, but recovered shortly afterward.

The next worker was Wollstein (1916, 1918, 1921) who reproduced parotitis, orchitis, and meningitis in cats

Important results were obtained by Johnson and Goodpasture (1934, 1935, 1936 *a*, *b*) After instillation of saliva into Stensen's ducts of rhesus monkeys a definite parotitis developed. These workers were able to passage the virus for over 10 generations. A number of other authors has been able to isolate a similar virus from cases of mumps (e.g, Findlay and Clarke, 1934, Levaditi *et al*, 1935 *a*, *b*, *c*, Lavergne *et al.*, 1937 *a*, *b*, and see below).

PROPERTIES OF THE MUMPS VIRUS

Human Inoculation

Johnson and Goodpasture (1935) sprayed a suspension of infected monkey's salivary glands into the nostrils and toward the Stensen's ducts of 13 susceptible persons. In 6 cases definite clinical mumps developed, after an incubation period of 18 to 33 days. Monkey-passed virus was used by Stokes *et al* (1946), and introduced in the diet or sprayed over the buccal mucosa, infection was characterized by pyrexia, enlarged parotids, a red orifice to the ducts, headache, and nausea It has also been reported that a laboratory attendant developed mumps after working with the virus (Levaditi *et al*, 1935 *c*).

Animal Experiments

Monkeys.

The following monkeys are susceptible to the virus bonnet, rhesus, and cynomolgus, chimpanzees are very susceptible (Levaditi *et al.*, 1935 *a*) Ceropithecus and cynocephalus monkeys are insusceptible to intraparotid injection (Levaditi *et al*, 1935 *c*). Using intracerebral injection, Gordon (1914) found that cynomolgus monkeys are more susceptible than rhesus Using intraparotid injection, however, it would appear that rhesus monkeys are perfectly satisfactory.

1. *Intracerebral injection* A proportion of monkeys develops meningo-encephalitis after intracerebral injection of infective material (Gordon, 1914, 1927, Lépine, Sohier, and Sautter, 1940, Swan and Mawson, 1943) Virus may be injected intraperitoneally as well to render the results even more certain A day or so after injection the animals become quiet and hang their heads, if touched they are irritable, and the limbs may be rigid In one case Gordon (1914) carried out lumbar puncture and found a pleocytosis of 15,000 cells per c mm with 82 per cent. of lymphocytes.

The animals usually die by the 4th day The pathological features have been described by Gordon (1914) Naked-eye, the meninges, brain, and cord appear congested. Histologically, the pia is infiltrated with lymphocytes The substance of the brain and cord is congested and the gray matter shows petechiae The ante-

rior horn cells may show degenerative changes, and although neuronophagia is not present there is some reactionary gliosis. The cerebral cortical nerve cells may also appear somewhat degenerate. Prolonged search may reveal an occasional focus of perivascular cuffing and more diffuse lymphocytic infiltration in the cortex (Gordon, 1927).

2. *Parotid injection* The virus may be injected into the substance of the gland, through the skin in front of the ear (Nicolle and Conseil, 1913 *a, b*). Alternatively, Stensen's duct may be cannulated and about 2 c c of virus instilled (Findlay and Clarke, 1934, Johnson and Goodpasture, 1934; Levaditi *et al*, 1935 *a*) Immediately following instillation of the duct the gland becomes enlarged, but returns to normal in 2 to 4 days A transient rise of temperature may be noted about 3 days after inoculation. Such changes should not be confused with the significant and specific alterations which occur from the 6th to the 8th day onward. The temperature rises, the blood shows a leukopenia with relative lymphocytosis, and the parotid

The parotitis and tem-

oted that a severer and more general reaction developed, the animals were drowsy, listless, but easily excited The glands were markedly tender.

Later workers have confirmed the susceptibility of monkeys to introduction of virus (human, monkey, or egg) by cannulation of the duct, or direct injection

943, Habel,

1946).

been fully

described (Gordon, 1914; Findlay and Clarke, 1934, Johnson and Goodpasture, 1934, 1936 *b;* Levaditi *et al*, 1935 *a, b, c;* Bloch, 1937). To the naked eye there is considerable edema of the neighboring tissues, and the parotids are enlarged and hemorrhagic.

Histologically, the lesions are of a focal nature. The acini are primarily attacked, and early degenerative changes of the epithelium are shown by swelling and detachment from the basement membrane, the cytoplasm is granular and the nucleus hyperchromatic. Later, the cells become necrotic and mononuclear phagocytes and leukocytes fill the acinar lumen, to constitute a small inflammatory focus. The acinar changes are definitely prior to those in the interstitial tissues, where a serofibrinous and cellular exudate is found, with some petechiae, most marked in the region of the acini, ducts, and vessels The interacinar spaces are widened by the interstitial edema. The cellular elements found in the acini and interstitium are lymphocytes and mononuclear phagocytes, polymorphs are not found It must be emphasized that these acinar changes are focal, and examination of any one slide shows areas at different stages of involvement. The stage of recovery is manifest by disappearance of the interstitial exudate and edema, but an increase of the periductal cellular infiltration The affected acini heal by absorption of debris, without fibrosis.

Inclusion bodies have been described in the acinar cells (Johnson and Goodpasture, 1936 *b*), these are eosinophilic granules measuring up to 4 μ in diameter, and are best seen in cells presenting only swelling In cells showing early necrosis they are smaller and may be multiple.

It has been pointed out by Levaditi *et al* (1935 *b, c*) that normal saliva injected into Stensen's duct produces changes of the same nature as those just described However, the changes produced by mumps salivary specimens are more marked than those produced by normal specimens

3 *Intratesticular injection.* Findlay and Clarke (1934) injected the tunica vaginalis of two rhesus monkeys with infective material Histologically, the tunica was infiltrated with lymphocytes and plasma cells and some of the seminiferous tubules showed degenerative changes

4. *Other routes of injection.* Monkeys may develop parotitis after intravenous (combined with intraperitoneal) injections.

Rabbits.

Granata (1908 *a*, *b*) claimed to have infected rabbits by various routes A rise of temperature followed intravenous and intraparotid injections, and a local swelling followed the latter. Corneal inoculation also gave a positive result, small elementary-body-like granules being found in the epithelial cells These results cannot have been generally confirmed as little or no mention of the susceptibility of rabbits appeared until the work of Lavergne *et al.* (1937 *a*, *b*, 1938, Lavergne and Kissel, 1938). These workers claimed to have found the virus in the cerebrospinal fluid of human cases of mumps meningitis, and in other cases in the incubation period After intrathecal injection rabbits developed a pleocytosis in the cerebrospinal fluid Histologically, lesions were noted in the brain and cord Perivascular cuffing, diffuse and focal cellular infiltration, and diffuse demyelination all occurred

Cats.

Positive results have been recorded by Wollstein, particularly with half-grown animals (1916, 1918, 1921). After intraparotid injection of infective secretion, the glands become swollen in 6 to 7 days, a rise of temperature and leukopenia with relative lymphocytosis accompany this swelling. The virus can be found in the saliva of infected animals Histologically, the gland shows edema of the connective tissue with mononuclear infiltrations, the acinar epithelium is swollen

Intratesticular injection produces a swelling, with accompanying temperature and blood changes, after 6 to 7 days. Histologically, the epithelium is degenerate and there is imperfect spermatogenesis. The interstitial cells are swollen and there is a cellular infiltration between the tubules.

Following intrathecal injection, an aseptic meningitis develops, and lasts for 3 to 5 days The virus is demonstrable in the cerebrospinal fluid Histologically, the changes are slight, the pia is somewhat cloudy, and the vessels congested

Insusceptible animals.

It has not been shown that dogs are susceptible, although this was suggested by Busquet and Laveran (1897). Numerous other animals must have been used in the course of experimental work, but no positive results have been reported

An Interference Phenomenon

It has been reported that a cerebral inoculation of virus in mice interferes with the proliferation of WFI virus (Vilches and Hirst, 1947)

Antigenic Structure

No evidence has been adduced to suggest that different antigenic types of virus exist (Beveridge and Lind, 1946) The virus occurs in the form of elementary bodies associated with infectivity, hemagglutination, and some CF property, and there is also a soluble smaller component retaining some complement fixing power only (see below, Henle, Henle, and Harris, 1947).

Morphology and Filtrability

No definite elementary bodies have been described, but eosinophilic inclusions measuring up to 4 μ have been described in the cytoplasm of acinar cells in the parotid (Johnson and Goodpasture, 1936 *b*)

The virus traverses Berkefeld filters British, V and N (Granata, 1908 *a*, *b*, Gordon, 1914, Findlay and Clarke, 1934, Johnson and Goodpasture, 1934, Levaditi *et al*, 1935 *b*), but it does not pass Chamberland L, filters (Levaditi *et al*, 1935 *b*) Habel (1945 *b*) estimated the size at over 340 mμ

Reaction to Physical and Chemical Agents

The virus is destroyed in 1 hour at 55° C. (Gordon, 1914); Habel (1945 *b*) found 20 minutes sufficient for inactivation It resists freezing for at least 3 weeks, and desiccation from the frozen state for several weeks (Johnson and Goodpasture, 1934; Enders *et al.*, 1945) The virus resists glycerol for several weeks (Findlay and Clarke, 1934; Johnson and Goodpasture, 1934) It is inactivated by formalin, ether, and UVL (Habel, 1945 *b*, 1946).

Infection in the Fertile Egg

A number of workers has found that virus from human material or passed in monkeys grows readily on inoculation in the egg It can be introduced by the following routes, the presence of infection being judged by the occurrence of hemagglutination with yolk or amniotic fluid (see below), or the presence of complement fixing antigen.

1. After inoculation in the yolk sac, virus proliferates locally, but is found most abundantly (after 6–7 days incubation at 34–35° C) in the amniotic fluid, amorphous granules up to 0 5 mm may be found in the yolk (Levens and Enders, 1945, Habel, 1945 *b*, 1946, Beveridge and Lind, 1946, Beveridge, Lind, and Anderson, 1946, Burnet, 1946). Transfer can be carried out with yolk sac suspension or amniotic fluid. The amniotic fluid has a smoky appearance that can be removed by adsorption with fowl cells, and high-speed centrifugation (Beveridge, Lind, and Anderson, 1946).

2. The amniotic route is probably the most susceptible, and can be used for the isolation of virus from human salivary specimens (Habel, 1945 *b*, Beveridge, Lind, and Anderson, 1946, Burnet, 1946, Henle, Henle, and Harris, 1947).

3 Egg passage virus causes infection by the allantoic route; the allantoic fluid produces hemagglutination and contains CF antigen (Habel, 1945 *b*, 1946, Beveridge, Lind, and Anderson, 1946, Henle, Henle, and Harris, 1947). Habel (1946) showed that when allantoic fluid is frozen and thawed, part of the virus adheres to the urate deposit.

The injection of polysaccharide prevents virus growth (Ginsberg, Goebel, and Horsfall, 1947).

4. The chorio-allantoic route can be used, but no definite lesions are produced on the membrane (Rocchi, 1943, Levens and Enders, 1945).

On continued egg passage, virulence for the monkey may lessen (Enders *et al*, 1946 *b*)

HEMAGGLUTINATION

Sources of Hemagglutinin

Mumps hemagglutinin (HA) is found most readily in amniotic fluid after amniotic or yolk sac inoculation (Levens and Enders, 1945, Beveridge and Lind, 1946, Beveridge, Lind, and Anderson, 1946, Burnet, 1946) It can also be detected in yolk fluid (Beveridge, Lind, and Anderson, 1946), and allantoic fluid (Beveridge, Lind, and Anderson, 1946, Burnet, 1946) It occurs in much higher titer in egg fluids than in the allantoic and amniotic membranes (Henle, Henle, and Harris, 1947)

Monkey parotid gland virus does not agglutinate fowl cells, although complement fixing antigen is present (Levens and Enders, 1945)

Red Cells Susceptible

Mumps virus agglutinates fowl cells Beveridge and Lind (1946) found that when the same virus preparation was tested with cells from different fowls, the titers varied from 25–100, the differences in titer were more marked with weak preparations The same workers found that suspensions of human cells gave titers

of 30–50 with a suspension titering 300 with fowl cells, several guinea-pig cell suspensions gave titers comparable to those with fowl cells, pigeon cells were agglutinated to a lower titer than fowl cells.

Technique of Hemagglutinin Titration

Beveridge and Lind (1946) use 0.25 c.c. volumes of virus diluted in twofold steps, with an equal volume of 2 per cent. fowl cells, and finally 0.25 c c. of saline. The tubes are shaken thoroughly, and the tests read when the agglutinated cells have settled to the foot of the tube, at room temperature. The end point is taken as the dilution giving a central button of agglutinated cells surrounded by a thin even zone of agglutinated cells.

Properties of the Hemagglutinin

1. HA is adsorbed by fowl cells, and after 3½ hours at 37° C. is mostly eluted, the red cells are lysed during the elution process (Beveridge and Lind, 1946). Mumps virus does not elute so readily as Newcastle Disease Virus (Burnet, 1945)

2. Human cells treated with mumps virus in allantoic fluid of relatively low titer adsorb virus, subsequently most of the virus can be eluted, leaving relatively unstable cells, after washing, these cells can be stabilized (rendered inagglutinable) by the addition of antiserum (Burnet, 1945, 1946). Such stabilized cells cannot be agglutinated by mumps virus, but are susceptible to agglutination by NDV and influenza virus, cells treated with NDV or influenza viruses are not agglutinated by mumps virus (Burnet, 1945, 1946; Burnet, McCrea, and Stone, 1946). Mumps falls into the same hemagglutinative group as NDV and influenza, and is placed first in the linear series (see Ch LXI).

3. HA is destroyed at 56° C in 20 minutes, but CF activity is not affected (Beveridge and Lind, 1946).

4 The addition of 0.1 per cent formalin causes only a slight fall in titer for up to 7 hours, but a marked drop after 24 hours, amniotic fluid with up to 0 2 per cent. formalin shows no fall in titer after 4 weeks in the refrigerator (Beveridge and Lind, 1946)

5 Untreated amniotic fluid maintains its titer for several days in the refrigerator, but at 37° C there is some fall after 4 hours (Beveridge and Lind, 1946).

6 Etherization reduces the HA titer (Beveridge and Lind, 1946).

7 The bulk of the hemagglutinin and some of the complement fixing antigen are removed by centrifugation at 15,000 r p m. for 1 hour or at 20,000 r p m for 20 minutes, it is probable that the HA is the virus particle itself, and that some of the CF antigen is likewise virus particle, there being also a soluble CF antigen (Beveridge and Lind, 1946, Henle, Henle, and Harris, 1947).

Hemagglutination-Inhibition Tests

Specific antisera inhibit hemagglutination (Levens and Enders, 1945) In these tests, Beveridge and Lind (1946) use 5 times the concentration of virus needed to give the end-point, the virus is formolized. Guinea-pig cells give results easier to read, first, the serum under test (diluted 1/10) must be treated with 10 per cent. packed cells to absorb natural hemagglutinin for guinea-pig cells.

Sera from mumps convalescents may inhibit agglutination to titers of 80–320 (Beveridge and Lind, 1946).

HA-inhibiting antibodies develop in monkey's sera after the inoculation of live (egg) virus, these appear 2 days after complement fixing antibodies and reach their maximum titer 6 days after these antibodies, after 81 days HA-inhibiting antibodies are still present to high titer (Enders *et al*, 1946 *b*) It appears therefore, that the CF and HA antigens are not completely identical

Inhibiting antibodies develop in rabbits after one intravenous injection of virus (Beveridge and Lind, 1946)

Nonspecific Inhibition of Hemagglutination

Normal allantoic fluid, yolk sac and chorio-allantoic suspension, and amniotic fluid from 13–16 day embryos inhibit HA, especially by weak preparations of virus; the inhibiting substance cannot be destroyed in infected material without damaging the agglutinin (Beveridge and Lind, 1946). A similar inhibition is exerted by infected allantoic and amniotic membrane suspensions (Henle, Henle, and Harris, 1947).

Normal rabbit or human serum may inhibit agglutination; heating of human serum at 60° C. for 20 minutes, or rabbit sera at 62° C. for the same time, considerably reduces the nonspecific inhibition titer, without affecting the specific antibody (Beveridge and Lind, 1946). Friedewald, Miller, and Whatley (1947) found that inhibition was produced by normal serum and tissue extracts. Extracts of red cells that are agglutinable by the virus will inhibit agglutination, presumably by competitive combination of the extract with the virus (Bovarnick and de Burgh, 1947, Friedewald, Miller, and Whatley, 1947, de Burgh *et al*, 1947). Cells treated with polysaccharide are not agglutinated (Ginsberg, Goebel, and Horsfall, 1947).

Agglutination of Red Cells by Antiserum

Burnet (1946) found that human red cells treated with potent virus in amniotic fluid could be only partially stabilized, and such cells were agglutinated to high titer by the sera of convalescents. Red cells were prepared by adding 0 2 c.c. of packed washed human cells of group O to 5 c c. of mixed amniotic fluid and saline The tube was placed in a water bath at 37° C for 3 hours, and left overnight in the refrigerator. The cells were then resuspended, centrifuged, and the supernatant discarded. The cells were made up to 2 per cent in saline, and tested at 37° C for stability and agglutinability by a known active serum. Suitable agglutinable suspensions could also be prepared using allantoic fluid, but this proved more difficult The tests were made with 0 5 per cent suspensions of virus-treated cells Doubling dilutions of serum were made in calcium saline and an equal volume (0.25 c c.) of treated cell suspension added. Readings were made after 2 hours in a 37° C incubator by studying the pattern of the deposited cells

Marked antibody rises occurred in convalescence and on active immunization. The titers were higher, but the degree of response was in general similar to that shown by parallel hemagglutination-inhibition tests It seems probable that the agent in mumps virus sensitizing the cells to the agglutinating action of antibody is the virus itself, or an antigenically active derivative thereof.

IMMUNITY REACTIONS

Antigen-Antibody Reactions

The interaction between mumps antibody and antigen can be demonstrated by various techniques

Virus neutralization.

Sera of man, monkeys, and cats neutralize the infectivity of mumps virus on recovery from infection. This neutralizing effect has been demonstrated by inoculating cats with virus-serum mixtures (Wollstein, 1916). Monkeys can also be inoculated with test mixtures (Johnson and Goodpasture, 1934), the parotids can be tested for the quantity of CF antigen produced (Enders *et al*, 1945). Eggs may be inoculated in the allantoic cavity or yolk sac, infection being judged by the presence of CF antigen in the egg fluids when tested with known sera (Habel, 1945 *b*, 1946).

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Inhibiting antibodies develop in rabbits after one intravenous injection of virus (Beveridge and Lind, 1946)

The skin reactions were read at 24 and 48 hours, and the mean value of the measurements in 2 directions recorded

1 Mumps appeared most often in those persons who previously gave a negative skin reaction. Of 89 cases of mumps 75 per cent. occurred in those who exhibited no reaction after 48 hours Of those who reacted, half exhibited a zone of erythema not exceeding 10 mm in diameter

2 Confirmatory of these findings were the results of tests carried out in the first 5 days of an attack of mumps in 13 adults and 27 children, over 90 per cent gave negative skin tests

3 In a number of groups exposed to infection, it was possible to estimate approximately the relationship between the size of the skin reaction and the risk of contracting infection With a negative or weak reaction at 48 hours (diameter 10 mm. or less), the chances were from 1 in 3 to 1 in 6 that the person would develop clinical signs of mumps With a reaction from 11-15 mm in diameter, the chances were 1 in 13, with reactions from 16-25 mm the chances were about 1 50 One may, therefore, regard persons showing a skin reaction of less than 11 mm. as presumably susceptible, and those with a larger lesion as presumably immune

4 It seemed that when the incidence of positive reactors in groups exposed to mumps was less than 50 per cent, secondary cases were fairly numerous When the incidence was over 50 per cent, secondary cases were relatively uncommon

5 Skin tests were carried out in 529 adults and 306 children not known to have had mumps recently There was approximately twice as great an incidence of negative reactors in the children as in the adults There was a lower incidence (63 per cent) of the skin reactors in adults confined in institutions than in students (72 per cent), presumably because of the decreased exposure of the former group The reactions in adults tended to be larger than in children, at 48 hours

6 As the work progressed it became evident that readings taken at 48 hours were the more reliable, reactions proving weak at 24 hours tended to become positive by 48 hours, further, reactions due to the control material were much less commonly seen at the later period

7. On the whole, it appeared that antigen prepared from the monkey parotid was more satisfactory than infected amniotic fluid

8 In 82 per cent of 179 adults giving a history of mumps, the skin reaction at 48 hours was positive (11 mm and over), 9 per cent were negative, and 9 per cent showed reactions under 10 mm Of those giving negative histories, 58 per cent were positive by the skin test, presumably due to subclinical infection

For various reasons, it was not possible to ...
it appeared
adults, there

9 Nearly ... whose serum contained complement fixing antibody could be detected, by the simpler procedure of skin testing, the skin test was more sensitive than the complement fixation test Thus 57 of 111 adults giving a negative fixation test showed positive skin tests It appeared that the accuracy of the skin test in revealing the presumably immune individual was not as great in children as in adults There was a larger proportion of failures to show a positive skin test in those where antibody was present in the serum It is probable that on recovery from an apparent or a subclinical attack of mumps, serum antibody develops before ...

10 The develop...
or caused appearanc...
negative skin tests d...
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11 Observations on small numbers suggested that the intradermal inoculation of antigen might induce resistance to attack

Active Immunity on Recovery from Infection

Experimental.

Cats recovered from infection are resistant to further attack (Wollstein, 1916). Monkeys are also resistant (Johnson and Goodpasture, 1934) Monkeys in contact

Complement fixation.

Complement fixing antigen can be found in the following infected materials

1. It occurs in monkey parotid gland (Enders and Cohen, 1942, Habel, 1945 *b*, 1946, Beveridge, Lind, and Anderson, 1946). It appears within 4 days of infection, and reaches maximum concentration before the development of parotitis (Enders *et al*, 1945).
2. It is found in yolk sac (Levens and Enders, 1945, Habel, 1945 *b*, 1946, Beveridge, Lind, and Anderson, 1946); in amniotic fluid (Levens and Enders, 1945, Habel, 1945 *b*); in the chorio-allantois (Levens and Enders, 1945), and in allantoic fluid (Habel, 1945 *b*; Beveridge, Lind, and Anderson, 1946).

The relatively purified aqueous phase of ether-extracted yolk sac virus contains the same titer of CF antigen as the original, the antigen is preserved by ether or UVL (Habel [illegible]
serum, CF an [illegible]
1946) CF an [illegible]
preserved in the frozen state (Enders *et al.*, 1945).

Antigenic components.

The work of Henle, Henle, and Harris (1947) has shown that complement fixation depends partly on the EB and partly on a soluble antigen

1. In infected eggs, they found complement fixing titers to be higher in membranes than in fluids. There was a discrepancy between complement fixation, infectivity, and hemagglutinating capacity. Both the latter properties were better developed in the fluids.
2. By centrifuging egg fluids for 20,000 r.p m for 20 minutes the infective, hemagglutinating and complement fixing properties were sedimented together with virus particles After centrifuging egg membrane suspensions, however, the supernatant still fixed complement. This soluble antigen was largely removed by further centrifugation at 30,000 r p.m
3. When human convalescent serum was absorbed with virus particle antigen from allantoic fluid, it still fixed complement with the soluble antigen. Absorption of serum with soluble antigen from membrane suspension left antibodies to the virus particle antigen
4. Antibodies against both antigens were found in the sera of children convalescent from mumps. Some sera from individuals giving a long past history of mumps showed antibodies only against the virus particle antigen

Evidently, therefore, one complement fixing component (found especially in egg fluid) is closely linked with the virus The other, found chiefly in membranes, is smaller and soluble

Other reactions.

Antigen-antibody reactions involving the red cell have been discussed above.

Skin Reactivity

Reagents can be prepared from infective materials that elicit a skin reaction in persons who give a previous history of mumps (Enders, 1945). The reagent is prepared from monkey parotid gland and inactivated by heat (Enders, Cohen, and Kane, 1945), or yolk sac antigen can be used (Habel, 1945 *b*).

Reactivity appears 1 week to 3 months after onset, and is unrelated to the development of complement fixing antibodies (Enders, Cohen, and Kane, 1945)

Enders *et al* (1946 *a*) carried out skin tests with a saline suspension of monkey parotid gland inactivated at 65° C for 20 minutes, and preserved with 0 5 per cent. phenol. In some experiments, suspensions of infected amniotic sacs were used

1942; Beveridge, Lind, and Anderson, 1946, Enders *et al*, 1945, 1946 *b*, Habel, 1946). They develop in 10–12 days, reach a maximum at 14, and then decline, some antibody persists for months

In man.

Virus neutralizing antibodies develop in convalescence (Johnson and Goodpasture, 1936 *a*, Enders, 1945, Enders *et al.*, 1945). Antibodies to the particle and soluble antigen may occur separately (Henle, Henle, and Harris, 1947).

Complement fixing antibodies appear in convalescence after about 2 weeks, or if already present, increase in titer (Enders and Cohen, 1942, Enders, Cohen, and Kane, 1945; Beveridge, Lind, and Anderson, 1946) CF antibodies develop after about 2 weeks if virus is inoculated in Stensen's duct, and in about 3 weeks if virus is sprayed (Stokes *et al*, 1946). Complement fixing antibodies develop as a result of subclinical infection (Enders, Cohen, and Kane, 1945).

Maris *et al.* (1946) made a detailed study of the antibody response, using the CFT, with monkey parotid as the antigen: 1. 56/61 cases of parotitis studied appeared in persons giving a negative CFT. Only 1 case occurred among 163 giving positive tests With rare exceptions, a positive CFT indicated resistance.

2. Up to ⅔ of exposed persons in institutions where mumps is occurring may develop a rise in CF antibody from subclinical attack.

They found the CFT positive in 42 per cent. of 386 persons giving no history of mumps. About ⅓ of all infections may be inapparent; inapparent infection occurs in childhood also.

3. The CFT was found to be positive in 77 per cent. of 565 persons giving a history of attack.

4 A titer of over 1/192 was regarded as presumptive evidence of a recent, as opposed to a remote, infection

5. CF antibodies were found in the sera of infants, and the results corresponded with those in the mothers

Serum Antibodies after Vaccination

Experimental.

Virus neutralizing and complement fixing antibodies develop after injection of live or inactivated vaccines and reach a level comparable to those in animals convalescent from infection (Habel, 1946). The presence of complement fixing antibodies does not necessarily mean that the monkey is resistant to infection (Enders *et al*, 1945).

The antibody response is higher when vaccines are incorporated in beeswax oil as an adjuvant (Habel, 1946).

When immune monkeys were challenged with live virus, virus neutralizing antibodies developed to 1/100 or higher within 3 weeks of the challenge—definitely higher than in controls following 1 injection (Habel, 1946). Similar observations have been made with CF and hemagglutination-inhibiting antibodies (Enders *et al*, 1946 *b*).

In man.

Complement fixing antibodies appeared after vaccination with formolized monkey virus in 18/45 cases, 10 days after the first dose. The presence of antibody was usually associated with resistance, but in one resistant group no antibodies were detected (Stokes *et al*, 1946)

After 1 inoculation of infected amniotic fluid, 5/8 persons showed an appreciable rise in agglutination-inhibiting antibody, 4 of those who had a second injection showed no further increase. These 4 persons probably had a basal immunity,

with infected animals may develop resistance, presumably as a result of subclinical infection (Enders *et al.*, 1945).

In man.

Second attacks of mumps are very rare (see Rolleston, 1932). Resistance can be acquired by subclinical attack (Siegel and Camp, 1944), and antibodies develop in a considerable percentage of persons who give no history of mumps (see below).

Active Immunity from Vaccination

Experimental.

Johnson and Goodpasture (1936 *a*) carried out experiments attempting to immunize monkeys with live virus. They were able to induce active immunity by (*a*) causing a clinically evident infection of the parotids; (*b*) producing a subclinical infection, (*c*) spraying virus-containing material into the nose and throat; (*d*) an intracerebral injection.

Inactivated formolized and alum-precipitated formolized suspensions of infected monkey parotid were administered in monkeys by Enders *et al.* (1945). About 60 per cent. of vaccinated animals showed evidence of increased resistance to challenge, as indicated by a complete or partial suppression of the formation of CF antigen in the parotid glands. Later (1946 *b*), monkeys were inoculated with live egg virus, of lowered virulence, by 1 parotid duct. On the 95th day afterward, a challenge of live virus was introduced into the opposite gland The animals developed an accelerated response, with the production of a hard swelling on the 2nd day, which disappeared by the 7th. There was no facial edema This accelerated response appears to be an allergic phenomenon associated with the immune state.

Inactivated virus, prepared by etherization or exposure to UVL of allantoic fluid or yolk sac, given subcutaneously, in many cases protected against parotid swelling when the animals were challenged by the parotid duct (Habel, 1946).

In man.

Various attempts have been made at active immunization in man Levine (1944) sponsored an epidemic in a school, by not excluding contacts once the disease broke out Although about 50 per cent of all susceptible children developed the disease, several associated adults also contracted the infection. Leineberg (1945) recommended the intracutaneous inoculation of defibrinated blood removed in the acute phase

Stokes *et al* (1946) vaccinated children with inactivated formolized monkey parotid virus subcutaneously. They were challenged by the introduction of active monkey virus in Stensen's duct, or by spraying the buccal mucosa. About half the vaccinated children challenged by the parotid duct route developed an accelerated response in the parotids, and this was regarded as evidence of resistance. When the challenge was given by spray, there was no accelerated reaction, but the swellings were less or absent

Enders *et al.* (1946 *b*) sprayed live egg-passed virus of increasing virulence in the mouth at intervals of 8 weeks There was no enlargement of the salivary glands, and CF antibodies developed after use of first passage material.

Serum Antibodies in Recovery from Infection

Experimental.

Bloch (1937–8) could not demonstrate any antiviral action in the serum of monkeys, but Habel (1946) found virus neutralizing antibodies Complement fixing antibodies develop after injection of virus in the parotids (Enders and Cohen,

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because in 3 others a rise in antibody was only noted after a second injection (Beveridge and Lind, 1946).

LABORATORY DIAGNOSIS

To obtain specimens of parotid secretion, place swabs over the orifice of the ducts for about an hour, then collect about 3 c.c. of saliva; broth is added to the swabs and saliva. The material is then inoculated in monkeys via the duct, penicillin being added to reduce contamination Yolk sacs can also be inoculated with saliva, using penicillin and sulfonamide, the amniotic cavity is also suitable (Beveridge, Lind, and Anderson, 1946). Leymaster and Ward (1947) recommend the use of saliva mixed with penicillin and streptomycin and inoculated amniotically in 7–8 day embryos.

The main occasions on which laboratory methods may be of help are in the diagnosis of manifestations other than parotitis, particularly those in which no parot[illegible]
may [illegible]
with [illegible]
diagnostic value in any suspected manifestations of mumps. In convalescence, a rise in antibody may enable a retrospective diagnosis to be made (see above)

SERUM PROPHYLAXIS AND THERAPY

A number of authors has used convalescent serum to prevent mumps. Some benefit has been claimed, although it is not as effective as certain other convalescent sera (e g., measles). Regan (1925), for instance, stated that serum (2 to 4 c c.) given before the 7th day after exposure was effective. Zeligs (1932) made a similar observation, also Cambessédès (1933) with larger (10 to 20 c.c.) doses Gunn (1932) did not find the results very satisfactory, but considered a further trial was indicated More recent workers have obtained results that indicate some value (Kutscher, 1940, Lyday, 1941, Wesselhoeft and Walcott, 1942). Hepatitis may follow the use of pooled blood products (Beeson, Chesney, and McFarlan, 1944, McFarlan and Chesney, 1944, McFarlan and Dible, 1944, Neefe, Miller, and Chornock, 1944) The injection of whole blood has been commented on favorably (Hess, 1915, Barenberg and Ostroff, 1931).

Convalescent serum has been given to males with parotitis in an attempt to lower the incidence of orchitis, it has also been used therapeutically The results in either case appear conflicting (Rambar, 1944, 1946, Candel, Wheelock, and Grimaldi, 1945, R. G Smith, 1945) However, gamma globulin prepared from convalescent blood lessened the incidence of orchitis from 24 7 per cent. in controls to 7 8 per cent (Gellis, McGuinness, and Peters, 1945)

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CHAPTER XXV

CHICKENPOX (VARICELLA)

CLINICAL FEATURES

Incubation period. This is relatively long, most cases occurring 13 to 17 days after exposure, although 11 to 20 days are the outside limits (Gordon and Meader, 1929).

Prodromal period. The onset of the disease is usually abrupt, although prodromata of vomiting, pyrexia, and erythematous rashes may be noted. Severe hemoglobinuria has been reported in a Jewish child (Kaiser and Bradford, 1929). Lade (1933) has pointed out that cases frequently have a diarrheal stool about a fortnight before the appearance of the rash.

The rash. This is usually the first clinical manifestation of infection, and only a brief description of its characters will be given here (see Rolleston, 1929, Rundle, 1929) Each individual spot of the rash passes rapidly through macular and papular stages to vesicle formation. In certain cases pemphigoid bullae may develop (see Gautier and Thévénot, 1929, Glanzmann, 1936). The vesicles are frequently oval and domed, being situated superficially in the skin; they are generally discretely spaced When the rash occurs on the hands, feet, and scalp the vesicles feel distinctly "shotty," being situated more deeply in these areas The vesicle usually collapses when pricked, with the escape of clear serous, or later slightly turbid, fluid, in certain cases the contents may suppurate. After a few days the lesions begin to heal, the vesicles rupturing spontaneously to form a blood-colored scab In certain cases the vesicle may not collapse entirely, giving an umbilicated scab The separation of deeper scabs may leave white scars which persist for life.

With regard to distribution, the spots are generally noted first, and later most profusely, on the trunk, in contrast to smallpox the extremities are not heavily involved The face is often severely affected and vesicles are usually found on the mucosa of the oral cavity and palate In severe cases stomatitis, laryngitis, and nasal lesions may occur (Millous, 1936). The vesicles usually break out in several crops at intervals of a few days Some days after the onset of the eruption the typical chickenpox patient shows vesicles, scabs, and scars. Associated with the spots there is usually considerable itching General symptoms are slight, a rise of temperature 3 to 4 days after the onset being quite common if the vesicles suppurate

It is well known that various forms of skin irritation serve to localize the distribution of the rash Thus Rivers and Tillett (1923) showed that the rash occurred most profusely under strips of adhesive tape, napkins, soft collars, or on faces affected by acne. Following the intradermal injection of streptococcal toxin the rash was found to be more profuse in that area (Martmer, 1927) Exposure to sunlight or ultraviolet radiation also tends to increase the number of spots in the irradiated area (Mallinckrodt-Haupt, 1934) In one interesting case it was found that the injection of diphtheria prophylactic (TAM) during convalescence resulted in the reappearance of a crop of vesicles on the gluteal, lumbar, and posterior femoral regions (Donald and Brosius, 1931)

Ronaldson and Kelleher (1938) reviewed various anomalous types of rash, illustrated by their own cases They allude, among others, to the following modifications (*a*) abortive rashes proceeding no further than the vesicular or even papular stages, (*b*) rashes retarded in their development in debilitated subjects, (*c*) gangrenous varicella (*vide infra*), (*d*) bullous varicella, (*e*) hemorrhagic varicella, (*f*) varicella herpetiformis

COMPLICATIONS

Although chickenpox is essentially benign, any large series of statistics shows

outbreaks may occur.

1. Surface Complications

The following lesions were those most commonly found by Wishik and Bullowa (1935), over half the total complications being those of the body surface. erysipelas and cellulitis, abscesses, lymphadenitis, and conjunctivitis Gangrene was found in certain cases, especially in the presence of cellulitis This gangrene often involved the superficial abdominal tissues (see also Nichols, 1934, Watson, 1934) Banks and McCartney (1937) recorded a case of gangrenous varicella due to a hemolytic streptococcus, and Lavergne *et al.* (1938) to a *Staphylococcus aureus* The latter also reviewed some 60 papers on gangrenous varicella. True diphtheria bacilli have also been isolated from gangrenous varicella (Warren and Sutton, 1915, Joe, 1918) *Hemorrhages may occur into the vesicles or surrounding skin (see* Tilley and Warin, 1938, Brander, 1940).

2. Other Pyogenic Complications

Otitis media, pharyngitis, laryngitis, tonsillitis, pneumonia, septicemia, appendicitis, and corneal ulcer have all been recorded as complications of varicella (*Mitchell and Fletcher, 1927, Bullowa and Wishik, 1935, Cunningham, 1943*).

mber of later writers (Loewen-

3illo, 1946). Irido-cyclitis may

thritis, myositis, bursitis, and

1927, Ochsenius, 1930, Tilley

1947)

3. Blood Diseases

Various types of blood disease have been reported as complicating varicella Thus, lymphatic leukemia has been recorded (see Goldman, 1930, Holbrook, 1937) Zoster eruptions may also occur in leukemia (see Ch XVI), and it is the *opinion of Philadelphy and Hasslhofer (1934) that* many such eruptions are in reality due to chickenpox.

Purpura may also occur in varicella (Fiessinger, Merklen, and Brouet, 1934, Cohen, 1936, Railliet and Ginsbourg, 1938) Fiessinger's case was one of hemorrhagic aleukemia, Cohen's of thrombocytopenic purpura, and Railliet's one of pronounced anemia.

4. Nervous Complications

mplications is not high (5 out of

cases have been recorded in the

925; Winnicott and Gibbs, 1926,

29, Butler, 1930, Fry, 1932, Gillot,

, 1932, Hallé and Arondel, 1933,

933, Imrie, 1934, Kimura, 1934,

Bernheim, 1935, Zischinsky, 1935, Chavany, 1937, MacIntyre and Beach, 1937, Walcott, 1937, Faust, 1938, Lancaster, 1938, Swyer, 1938). In addition there have appeared a number of general reviews with more or less extensive bibliographies (Fasella, 1929, Cornil and Kissel, 1930, Brain, 1931, Zimmerman and Yannet, 1931;

van Bogaert, 1933, Bergman and Magnusson, 1939, Deal and Gastelu, 1939, Masten, 1940; Waring, Neubuerger, and Geever, 1942, Machado, 1945, Swan, 1946).

The most comprehensive paper is that of Underwood (1935), who has reviewed some 120 published cases He has amplified various existing systems of classification and divided the nervous manifestations into 6 main groups. The clinical picture is variable, and the following are the main features which may be noted· convulsions and meningeal irritation; evidence of encephalitis, myelitis, peripheral neuritis, paralysis of the internal and external eye muscles.

Symptoms of nervous involvement may occur from the prodromal period up to 90 days later, but the great majority of cases develop symptoms between 4 and 10 days after the onset of the illness Lumbar puncture does not usually show any significant departure from normal, although cells may be increased up to about 30 per c.mm.

Underwood's series showed that 80 per cent. of cases recovered, 10 per cent died, and 10 per cent. were left with some permanent sequel, often some muscular paralysis or incoordination

The greatest number of cases of nervous involvement was probably recorded from 1925 to 1932. Many more cases seem to be recognized in France, Belgium, Italy, Germany, and America than in Great Britain.

Pathological features. van Bogaert (1930, 1932) found widespread encephalitis Areas of demyelination were noted, mainly in the centrum ovale, cerebellum, inferior peduncle, and to a lesser extent in the cerebral cortex, even outside these foci there was involvement of the axis cylinders These areas of demyelination were not invariably situated perivascularly. The basal ganglia were diffusely infiltrated with cells, and many ganglion cells in the cerebrum and cerebellum were degenerate.

Zimmerman and Yannet (1931) reported a widespread degeneration of ganglion cells in the brain and cord, in the cells of the cerebral cortex, central gray masses, pontine and medullary nuclei, cerebellum (Purkinje cells), dentate nucleus and both horns of the cord. The vessels of the parietal and occipital lobes were "cuffed" with fat-granule cells, and there was a perivascular zone of demyelination

A case in an adult was examined by Waring, Neubuerger, and Geever (1942); demyelination was present. Swan (1946) found diffuse and perivascular softening in the neostriatum. Perivascular demyelination was found in the white matter.

Etiology of nervous complications Underwood discusses at length the various possible explanations for nervous involvement, such as the coexistence of encephalitis lethargica, the action of an unknown virus, an anaphylactic reaction, but decides that the condition is in all probability due to the development of neurotropic tendencies in the strain of varicella virus itself Many cases of nervous manifestation resemble postvaccinal encephalitis both clinically and pathologically, and the etiology of these 2 conditions must be considered together A full discussion is to be found elsewhere (see Ch. XXXV), here we may state that in our opinion varicella virus is probably one of many agents causing a vascular reaction, characterized by the production of demyelination, in the central nervous system Possibly this reaction is of an allergic nature

5. Congenital Lesions

It is possible that congenital lesions may be attributed to maternal varicella in the first trimester, just as occurs in rubella (Ch. XXIII; Laforet and Lynch, 1947).

CLINICAL PATHOLOGY

The opsonic index (test organisms: *Bact. typhosum*, *Br melitensis*, and *Streptococcus*) was said to be considerably raised in varicella (Piana, 1930).

Although a leukocytosis usually occurs, the blood count is not of very much diagnostic value Mitchell and Fletcher (1927) showed that in infants the count was

often 3,000 cells per c mm above normal from the first day of the illness onward. Between the ages of 6 and 10 a similar increase occurred, but not until the 3rd day, while in adults it did not take place until the 4th to 9th day. These workers noted no significant change in the differential count. Holbrook (1941) refers to a leukopenia at the end of the incubation period. He found an increase in plasma cells early in the eruptive stage

HISTOPATHOLOGY OF THE VARICELLA VESICLE

The histopathology of the vesicle has been described and figured by various authors (see Unna, 1896, Dockerell, 1905; and Tyzzer, 1905–6; Dahl, 1944) and closely resembles that of zoster. The vesicle lies mainly in the prickle cell layer and is dome-shaped, the roof being formed by the stratum corneum and lucidum, and the broad base by the deeper prickle cell layer Although the cavity is subdivided by a few septa, it collapses when pricked The process of vesiculation begins by a reticulating liquefaction in a few prickle cells (Unna). These cavities fuse together to form vesicles which in their expansion compress nonliquefied cells to form septa In addition, ballooning degeneration takes place in the cells at the base, margins, and center of the vesicle These swollen cells, containing numerous nuclei, are usually

vesicle (Manicatide

fibrinous with some

congestion and perivascular multiplication of spindle cells Inclusion bodies have been described by Tyzzer (1905–6). They are eosinophilic or purple granules occurring first in the vascular endothelium and later in the epidermal cells. They are usually intranuclear but intracytoplasmic forms may occur, they measure from 1 μ to 6 μ in diameter.

VISCERAL LESIONS

Giant cells have been described in the tonsils in the prodromal period (Tomlinson, 1939).

Johnson (1940) examined the tissues of a child dying in the acute phase, and found areas of focal degeneration in the esophagus, pancreas, liver, renal pelves, ureters, bladder, and adrenals The cellular changes are like those in the skin. The affected cell is ballooned, and the cytoplasm vacuolated, the nucleus also is rarefied The capillaries associated with the lesions are often destroyed with thrombosis and hemorrhage. Johnson suggests that the virus propagates in the blood, localizes in epithelial cells and multiplies Inflammatory cell infiltration occurs secondarily to infarction and necrosis. Waring, Neubuerger, and Geever (1942) found "virus pneumonia," encephalitis, and toxic nephrosis in an adult

Oppenheimer (1944) examined a case of congenital varicella in a premature infant dying at 11 days. He found disseminated necroses with intranuclear inclusions in skin, esophagus, stomach, intestines, pancreas, lungs, bronchioles, kidneys, liver, adrenals, and thymus.

EPIDEMIOLOGY

Chickenpox is found in all parts of the world. Although the disease is predominantly one of youth, older persons do not escape entirely Thus, while the majority of cases occurs from 2 to 6 years, almost 20 per cent of cases are over 20 years of age (see Mitchell and Fletcher, 1927). The disease has been reported in babies aged 2 to 8 days due to infection *in utero* (Henderson, 1934, Baron, 1935, Shuman, 1939, Oppenheimer, 1944), the oldest case is that of a woman aged 76 (Rolleston, 1932)

In temperate climates the disease is somewhat more prevalent in the 6 winter months than in the summer time, but it does not enjoy any marked seasonal distribution

The disease is one of moderate infectivity and frequently causes difficulty in isolation hospitals, as the condition may smolder in infected wards for a considerable time. When originally introduced to any institution it may be very hard to trace the offender. No child with a frank varicelliform rash may appear to have been admitted, and one must postulate that some entrant had only a few vesicles which were not obvious on clinical examination. Alternatively, a case may have been admitted in the incubation period.

It is probable that varicella is infective from 24 hours before the eruption till at least 7 to 8 days after its appearance. Infection is spread by droplets and droplet nuclei or by contact with the rash. Fomites may play a slight part in the spread of the infection if used directly after being contaminated by the patient. Contacts should be quarantined for 21 to 24 days.

Favorable results, with lowered incidence of infection, have followed irradiation of children's wards and school rooms (Greene, Barenberg, and Greenberg, 1941; Wells, 1945).

Chickenpox in schools. The regulations (*Memorandum*) require cases to be excluded from school for 2 weeks from the appearance of the rash. Contacts need not be excluded (Forbes, 1939).

Chickenpox has been discussed in a special report of the Medical Research Council on epidemics in residential schools (1938). It was found to be the commonest infectious disease in boys and girls, but the attack rate on susceptibles was never a very high one. The epidemics usually lasted throughout the term

ISOLATION OF THE VARICELLA VIRUS

Although it has been suggested that the etiological agent of varicella is a cultivable micrococcus (Auricchio, 1924, Macdonald and Macdonald, 1931), it is generally held that it is a virus.

Human Inoculation Experiments

The infectivity of varicella was first shown conclusively by Steiner in 1875. He was able to reproduce varicellous eruptions by inoculating children with varicella vesicle contents. These observations have been confirmed repeatedly (Kling, 1913, 1915, Lapidus, 1916–18, Meyer, 1918–19, Gyr, 1916–18, Bigger, 1937). In addition other authors have carried out similar experiments, mainly to produce active immunity against the disease, and obtained local lesions (*vide infra*). It should be added that certain workers have failed to transmit the infection with vesicle fluid (Hess and Unger, 1918, Gulácsy, 1933; Wats and Dalal, 1937).

Animal Experiments

A number of workers have failed to transmit the infection to animals, the following having given negative results: monkeys by dermal inoculation (Park, 1902; Tyzzer, 1905–6, Gordon, 1925), guinea-pigs by intradermal injection, after tarring of the skin (Greenthal, 1926), rabbits by corneal inoculation (Salmon, 1905, Tyzzer, 1905–6), or dermal inoculation (Gordon, 1925). However, positive results have been recorded by various workers.

Rabbits. Rivers and Tillett (1923, 1924 *a, b*) injected rabbits with varicella fluid intratesticularly and obtained a definite reaction after a few passages. It soon transpired, however, that they were dealing, not with varicella virus, but with virus III.

Similar experiments were carried out by Taniguchi *et al.* (1932 *a, b*) who injected varicella vesicular fluid intratesticularly into rabbits, thus isolating 3 strains of virus. Taniguchi also injected rabbit passage virus into the anterior chamber of the eye. The cornea and iris became swollen, the aqueous humor turbid, and there was a slight conjunctivitis. Some time earlier it had been shown that eosinophilic inclusions developed in the superficial epithelium after corneal inoculation (Bertarelli, 1909, Swellengrebel, 1911, Gins, 1918), and Taniguchi described clusters of

elementary bodies in corneal endothelial cells. Intradermal injection produced a localized eruption There appears to be every reason for accepting Taniguchi's findings, as it has not been shown that his rabbits were infected with virus III[1]

Monkeys Rivers began to use monkeys, as his particular stock of rabbits was unsuitable for attempting the isolation of virus. He found that vervets (*Cercopithecus lalandi*) and green monkeys (*Cercopithecus sabaeus*) may be used for intratesticular injection (1926, 1927). After such injection large acidophilic granular inclusions form in the nuclei of the tubular cells The monkeys must be young (spermatogenesis must not have begun) and the testicles should be removed after 5 or 6 days

More recently, Eckstein (1933–4) has reported that rhesus monkeys may develop a fatal encephalitis after intracerebral injection of varicella fluid Histologically, thromboses, perivascular infiltrates, and degeneration of the axis cylinders and pyramidal tracts are found. If confirmed, this observation should lead to further progress in the knowledge of the virus and its properties

Morphology

The *elementary bodies* of varicella were probably first described by Aragão in 1911 Since that date they have been studied by a number of independent observers (Paschen, 1917, 1933–4, Taniguchi *et al*, 1932 *a*, *b*, 1934, 1935; Amies, 1933, 1934). The bodies are found in the vesicle fluid in the early stages before crusting begins. They can be purified by differential centrifugation (Amies, 1933), they are specifically *agglutinated by varicella convalescent serum (Amies, 1933), as well as by* zoster convalescent serum (see Ch. XVI). EB's are always smaller and scantier than those of variola (van Rooyen and Illingworth, 1944).

Further proof of the etiological nature of these bodies has been afforded by Taniguchi *et al.* (1932 *a*, *b*) These workers found that the bodies occurred, mainly in clusters intracellularly, in preparations of Descemet's membrane made after intra-ocular injection Also, they could be demonstrated in infected tissue cultures (1932 *a*) and egg membranes (1935). Further, egg membranes in which these bodies could be demonstrated were capable of inducing typical pocks on intradermal injection of children.

EB's have been found by the electron microscope, they are said to measure 210 x *238 mμ (Nagler and Rake, 1938).*

Inclusion bodies, as already mentioned, have been described by various authors (see also Simon and Scott, 1924). These bodies are to be found in the epidermal and vascular endothelial cells of the vesicular lesion, as well as in inoculated rabbits' corneae and monkeys' testes. These large eosinophilic structures are exactly similar to those described in zoster (see Lipschutz, 1921) and are usually found intranuclearly, as they have only been studied morphologically it is uncertain whether or not they represent the etiological agent. Taniguchi *et al*, have also described inclusions, which are intracellular aggregates of elementary bodies and may represent the etiological agent These aggregates have been found in the cells of Descemet's membrane after intra-ocular injection, as well as in the epidermis of the infected chorio-allantois, and tissue cultures.

Cultivation

The virus isolated by Taniguchi *et al.* (1932 *a*) was cultivated in the presence of rabbit lung and testis for at least 11 generations Elementary bodies were demonstrable throughout the passages.

Taniguchi *et al* (1935) used their virus to inoculate eggs and found that pocks

[1] In view of the failure of others to confirm Taniguchi's discovery, it is only fair to add that certain workers feel that his virus may be a strain of variola-vaccinia

developed on the membrane and sometimes on the surface of the embryo as well, elementary bodies could readily be seen in films made from these lesions. The chick membrane attained maximum virulence about the 5th to 6th day after inoculation, when 0 2 c c. of suspension, diluted 10^{10}, was capable of producing local lesions on *intradermal injection of rabbits. Cross* immunity tests failed to demonstrate any antigenic difference between egg virus ("ovo-vaccine") and testicular virus Proof that the agent grown was probably the causal agent of varicella was afforded by showing that after intradermal inoculation of "ovo-vaccine" 13 out of 15 susceptible children developed typical chickenpox vesicles. Teixeria (1936) also noted discrete pocks on the chorio-allantois. However, Irons *et al.* (1941) failed to produce recognizable lesions, and this seems to be the usual experience

IMMUNITY REACTIONS IN CHICKENPOX

(*a*) *Human.* Recovery from varicella is usually associated with a solid immunity to reinfection. It is generally agreed, however, that in a small percentage of cases second attacks do occur. Thus in 775 cases recorded by Mitchell and Fletcher (1927) 9 had had a previous attack (see also Lévy, 1933). Leak (1940) records 4 attacks in a boy aged 13. Relapses may occur, with a fresh eruption, within a short time of onset (Hughes and Smith, 1939).

With regard to the presence of immune bodies in varicella convalescent serum, agglutinins for the elementary bodies have been described (Amies, 1933), complement fixation occurs using varicella crust antigen (Netter and Urbain, 1926), even several years after the attack (Hasskó *et al*, 1938). Finally, serum-virus mixtures fail to produce characteristic inclusions on intratesticular injection of monkeys, presumably due to virus neutralizing antibodies (Rivers, 1926, 1927).

Attempts have been made to immunize children actively by injecting them intradermally, shortly after exposure, with fresh varicella vesicle fluid (Greenthal, 1926, Cherkasoff and Goodeen, 1929, Angarano and Gabrielle, 1931, Csagoly, 1932, Durand and Conseil, 1932). Convalescent whole blood has also been recommended (see Orosz, 1929, Siegl and Rupilius, 1929) It has been stated that the intradermal injection of blood removed during the first 48 hours of the rash prevents a high percentage of contacts contracting varicella (Barberi and Rubini, 1932) Taniguchi *et al* (1935) *have suggested that their "ovo-vaccine" is eminently* suitable for active immunization of children

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Tests for the differential diagnosis of chickenpox and smallpox are fully described elsewhere (see Ch XXVII) The obtaining of negative reports from the usual tests is of value in the diagnosis between confluent varicella and variola (Maddock, 1946). As far as chickenpox itself is concerned, films may be made from the vesicles and stained for elementary bodies (see Ch III) An attempt may also be made to isolate the virus by intratesticular injection of rabbits or monkeys

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CHAPTER XXVI

VARIOLA-VACCINIA AND ANIMAL POCK VIRUSES

THERE OCCURS in man and animals a number of diseases characterized by the development of pocks, these diseases bear definite resemblances one to another, both clinically and as regards the properties of their causal agents. The diseases which affect man are true smallpox (variola major), alastrim (variola minor), and cowpox. The diseases of animals definitely in this group are cowpox and rabbit-pox. Some workers include also pox diseases of horses, camels, sheep, goat, and swine, but the justification for this grouping is open to doubt By suitable animal passage, the "laboratory" virus of vaccinia can be produced from variola, alastrim, cowpox, and perhaps other sources.

The Origin of the Human and Animal Pock Viruses

Gordon (1925)[1] believes that vaccinia is the more primitive virus partly because it is clearly more comprehensive in its infectivity than variola, also because it is more potent antigenically than variola. Findlay (1936) advanced 2 theories to account for the relationship between the pock viruses First, the animal pock diseases, with variola and alastrim, may have evolved from a hypothetical primitive pox It cannot be postulated, however, in what host, possibly man, possibly animal, this primitive pox occurred. The second possibility is that human variola was the source of the animal diseases and of alastrim In this case man would have been the starting point, and the animal diseases presumably would have originated owing to domestication and contact with him Let us suppose that animals in close contact with man at one time developed an attack of variola It is not unreasonable to suppose that by continued transfer through the particular animal species the variola virus would have become adapted, and have produced a disease of different nature from the original

It does not seem possible, on the evidence available, to support any one of the 3 above-mentioned theories more than the others. The question is, however, largely academic and of no great practical significance.

The Relationship of Variola and Alastrim

The 2 diseases resemble each other closely, apart from the less severe nature of alastrim

The viruses have similar biological properties, but their inclusion bodies can be distinguished They appear to have a similar antigenic structure, as shown by the results of complement fixation, flocculation, and agglutination tests (Kryloff, 1911, Gordon, 1925, Amies, 1932) Cross immunity tests in monkeys have been carried out (Green, 1915–1917, Horgan and Haseeb, 1939) The latter found that immunity was not fully reciprocal, and that the degree of protection induced by alastrim was somewhat greater than that provoked by variola We may conclude that the 2 agents are very closely related, but whether alastrim is a variant of variola, or whether both are derived from a primitive pox is unknown (Ledingham, 1925, Gins, 1927, Kaiser, 1934, Findlay, 1936, Jorge, 1936)

The Relationship of Variola and Vaccinia

By passage through animals, variola virus from the human case can usually be adapted and altered to become vaccinia A similar result can be achieved by variolization or arm-to-arm transfer in man With regard to the nature of the change,

[1] References are appended at the conclusion of Ch XXXV, p 403 et seq

Findlay (1936) suggests that vaccinia is a stable variant of variola (or alastrim) which appears on transfer to a new host The particles of vaccinia are probably present in the original inoculum, and overgrow the variola particles in a selective environment The factors determining the change from variola to vaccinia are imperfectly understood, and under certain conditions variola virus proves stable. For example, variola passed on the chorio-allantois has not been found to develop the properties of vaccinia (Nelson, 1939, 1943; North, 1944).

There are a number of points of distinction between variola and vaccinia viruses For example, the greater infectivity of vaccinia for the calf, rabbit, and other animals, and differences in the inclusion bodies As regards the antigenic structure, there is a close similarity Kolmer (1916) using the CFT found the 2 agents to be closely related. In tests used in the laboratory diagnosis of smallpox, complement fixation is regularly obtained between variola crusts and antivaccinial serum Passive immunity experiments indicate a close antigenic relationship (Pandit *et al*, 1931–2). In cross immunity experiments, usually in monkeys, it has been found that vaccinia protects against variola more effectively than variola protects against vaccinia (Brinckerhoff and Tyzzer, 1906; Bachmann and Biglieri, 1923 *a*, Gordon, 1925, Horgan and Haseeb, 1939). There is little doubt that this is because vaccinia readily proliferates in the skin, and develops a large amount of immunizing material, variola, on the other hand, proliferates less readily. The larger the amount of variola virus in the skin, the greater will be the resistance to vaccinia (see Horgan and Haseeb, 1939) Gordon (1925) regards vaccinia as consisting of equal parts of variolar and vaccinial components Variola as freshly isolated contains only a small amount of vaccinial element, but this increases on passage Horgan and Haseeb (1939) support this view, and suggest that variola contains a group (vaccinial) antigen as well as a highly specific (variolar) antigen The group antigen is r [illegible]
for [illegible]
cor [illegible]
of [illegible]
man against infection by variola, and by the protection that variola confers against vaccinia

The Relationship of Alastrim and Vaccinia

The position is closely analogous to the relationship between variola and vaccinia By animal passage alastrim can be converted into vaccinia. Similar differences exist between the infectivity of alastrim and vaccinia viruses for animals, and the inclusions are not identical The antigenic structure as shown by complement fixation is identical, but the immunizing capacity of the 2 agents varies A number of workers has carried out cross immunity experiments, chiefly in monkeys (Green, 1915–1917), Blaxall, 1923, Gordon, 1925, Sobernheim and Zurukzoglu, 1928; da Cunha and Teixeria, 1934, Horgan and Haseeb, 1939, van Breuseghem, 1940)

These workers have shown that vaccinia immunizes more effectively against alastrim than the reverse This is because alastrim grows less abundantly in the skin than vaccinia It would seem reasonable to conclude that alastrim virus, like variola, consists of a basic vaccinal antigen and a specific alastrimic component.

Apparently certain observers in the Belgian Congo have thought that vaccinia may not protect against the local alastrim in human infections, and it may be that there are unusual local strains, however, the general experience is that *jennerian* vaccination immunizes effectively against alastrim (see Horgan and Haseeb, 1945).

THE NATURE OF COWPOX

Originally brought to prominence by the observations of Jenner, it is now well known that milkers and others may develop pocks on their hands after attending to animals suffering from cowpox The lesions in the human are closely *similar to*

those in the cow, and also resemble those produced by inoculation of vaccine lymph in ordinary vaccination.

Until recently the biological nature of the virus responsible for cowpox in man has been uncertain (Findlay, 1936; Gins, 1938). While some persons have regarded the viruses of cowpox and vaccinia as identical, others have held cowpox to be a virus *sui generis* in the same way as the viruses of classical smallpox and alastrim are so regarded It is probable that the latter view is correct, for Downie (1939 *a*) has shown that there are definite distinguishing features between the viruses of cowpox and vaccinia For instance, the experimental lesions of cowpox are the more hemorrhagic. The vaccinial lesions show a greater tendency to cellular necrosis, and polymorphs are more frequent in the exudate. The inclusions of vaccinia are irregular and granular, while those of cowpox are larger and more homogeneous In cowpox the inclusions are commonly seen in mesodermal cells, while this is less frequent with vaccinia; in cowpox, inclusions may also be seen in the endothelial cells of the small blood vessels. Serological observations also suggest that vaccinia and cowpox are distinct viruses (Davies, Janes, and Downie, 1938, Downie, 1939 *b*). Downie carried out cross absorption tests with EB's, and found that there were *qualitative differences in the heat-labile antigens of cowpox and vaccinia viruses.* Cross protection between cowpox and strains of vaccinia can be shown in rabbit experiments (Horgan and Haseeb, 1945). It is probable therefore that cowpox contains the group antigen found in vaccinia and variola as well as a specific component.

The Relationship of the Animal Pock Diseases

Findlay (1936), in a review of the literature, concluded that some, if not all, of the animal pock diseases mentioned in the first paragraph of this chapter could give rise to virus variants similar to vaccinia The position is, however, obscure and there is scope for considerable further investigation

There is no question that the viruses of cowpox and rabbit-pox (see p. 323) can be fairly regarded as belonging to the variola-vaccinia group. As regards sheep-pox, claims have been made that the causal virus can be converted quite readily into vaccinia (Gins, 1919, Toyoda, 1924, Kii and Kasai, 1926, Kasai, 1931). Rao (1938) using the egg membrane, however, could not produce typical vaccinia. More recently Bennett, Horgan, and Haseeb (1944) have investigated enzootic sheep-pox in the Sudan. They conclude that the causal virus is not related to the variola-vaccinia group. Gins (1919) claimed to have produced a vaccinial variant from goat-pox. Bennett and his coworkers found no immunological relationship between the causal agent and vaccinia, they found that goat-pox and contagious pustular dermatitis viruses shared antigenic components Gins (1927) claimed also to have produced vaccinia from swine-pox

It is evident therefore that later work does not tend to confirm earlier observations on the antigenic character of the causal agents of the pock diseases of some of the domesticated animals It would appear that the virus of ectromelia is closely related to vaccinia (see Ch XXXI)

VACCINIA VIRUS

As has been explained, vaccinia is a purely laboratory virus, of which a number of strains are in existence. It is probable that they have all derived from cases of variola, alastrim, or cowpox Horgan and Haseeb (1945) suggest that vaccinia viruses should be designated by their source, e g, variola-, alastrim-, or cowpox-vaccinia. They carried out cross immunity experiments between various strains of vaccinia, using chiefly dermal scarification in rabbits The strains tested were Khartoum, originally isolated from variola major, LMC (Amies) isolated in London from a case of alastrim, Nigeria, derived from the Lister Institute strain, mouse neurovaccinia (Lagos), neurovaccinia (Levaditi) Irrespective of the origin

of the strains, or animals used for the preparation of the immunizing or test dose, there was full immunological identity of all these strains of vaccinia A similar antigenic structure was found between a stock strain of vaccinia used for lymph production and a vaccinia strain originally deriving from variola (Pandit and Rao, 1940).

There is thus no evidence for the presence of antigenic types of the vaccinia virus. However a number of biological variants have been described (see Ch. XXVIII). Vaccinia has a complex antigenic structure consisting of antigens inherent in the EB, and a soluble (LS) antigen (see Ch. XXXIII).

CHAPTER XXVII

TRUE SMALLPOX, ALASTRIM, AND COWPOX

TRUE SMALLPOX (VARIOLA MAJOR)

In the discrete type, the pocks are few in number and scattered. This type includes cases referred to as "varioloid," often occurring in vaccinated persons, such cases of mild smallpox are capable of infecting nonimmune persons who may develop the severe confluent form of illness.

In the confluent variety the lesions coalesce in such a manner as to leave little or no healthy skin between them.

In those cases which present a combination of appearances the name coherent or semiconfluent smallpox is applied, and where hemorrhage develops the term hemorrhagic smallpox is used

Clinical Features

Nonhemorrhagic smallpox.

The incubation period is usually about 12 days, but may vary from 6 to 22 days in exceptional circumstances. The onset tends to be sudden, the illness being ushered in by pyrexia, headache, vomiting, flushing of the face, congestion of the conjunctivae, and furring of the tongue, with severe backache.

Prodromal rashes are often seen during this initial febrile period about the second day of the illness and, according to the character of the eruption present, have been divided into 3 varieties—erythematous, petechial, or mixed (Ker, 1920).[1] The erythematous form may be scarlatiniform, morbilliform, or multiform in appearance, and may be either general or local in distribution, but is said to avoid *the* face, it is rare in children under the age of 10. The scarlatiniform rash manifests itself as flushing of the skin and is usually distributed evenly over the body surface The morbilliform rash, Ker (1920) states, may closely imitate the rash of measles, with the exception that the macules tend to be larger and are not elevated above *the skin In addition, a multiform type of lesion, with both scarlatiniform* and measly characters, may develop. The petechial rashes are more common than the erythematous varieties, and Rundle (1929) remarked that he had noticed them in about 9 per cent. of his cases. They appear as a collection of punctuate red- or purple-colored spots which develop in the axillary or inguinal region In the latter situation, they may assume a highly characteristic distribution over the lower part of the abdomen, lying within a triangular area bounded above by a transverse line drawn through the umbilicus, laterally by the inguinal ligaments, the pubis being the apex, this condition is referred to by French clinicians as the "bathing drawers rash" (see Fig. 25) Lastly, in the mixed type of rash both the erythematous and petechial forms coexist Prostration is marked in the prodromal period

As the condition progresses, the patient becomes drowsy and restless, and may suffer from delirium and coma On the third day the specific eruption appears, commencing with the formation of small bright red macules, 24 hours *later these* develop into vesicles, which gradually become umbilicated, and increase in size to reach a diameter of 6 mm on the 5th or 6th day. Secondary infection of the vesicles then takes place and they become converted into pustules

The *distribution of the eruption in true smallpox is a characteristic* feature of the malady, and is of considerable diagnostic value. The pocks are most numerous on the face, forearms, wrists, palms of the hands, and soles of the feet, thereby

1 References are appended at the conclusion of Ch XXXV, p 403 et seq

conforming to the so-called centrifugal type of arrangement The lesions are less

detail, drew attention to the possible effect which friction or the intermittent pressure of clothing may exert (see also Wanklyn, 1913)

About the 9th day of the disease healing commences, and the pustular contents undergo absorption The pustules now gradually shrink, becoming converted into



Fig 25 Smallpox eruption as seen on second day of illness, affecting the inguinal region and referred to as the "bathing drawers" eruption At this and the later stage of vesiculation, elementary bodies are readily demonstrable *in tissue scrapings (see van Rooyen and Illingworth, 1944).*

flattened scabs which eventually drop off, to leave behind them an area of superficial discoloration which may disappear completely, if the lesions have not been deep-seated or the papillae damaged On certain parts of the body surface, such as the soles of the feet and palms of the hands, the pustules fail to erupt through the skin, and patches of discoloration remain visible after absorption of the contents Pocks may also form under the nails of the fingers and toes, giving pain

During the pustular stage of the disease, the patient's face becomes greatly swollen and edematous, the nose, cheeks, lips, and ears become thickened, there is difficulty in closing the eyelids and mouth, pus exudes from the conjunctivae, saliva dribbles from the mouth, and an offensive odor emanates from the body. Pocks may also appear on the tongue and larynx, swallowing becomes difficult,

and a painful cough may develop. These lesions are accompanied by severe constitutional symptoms, and drowsiness, delirium, and coma may become pronounced (see Fig. 26).

The temperature chart in a case of smallpox shows 2 rises; the first or pre-eruptive falls with the appearance of the rash, and the second, due to secondary pyogenic infection developing at the pustular stage, about the 8th or 9th day. The height of the fever ranges from 103° to 105° F., according to the severity of the case, and is accompanied by an increase in the pulse rate to about 120 beats per minute.

Infection of the fetus *in utero* may occur (Horning, 1932).

We do not intend to discuss the differential diagnosis of smallpox on clinical grounds, but would draw attention to the cardinal features of prodromal rashes



Fig. 26. Smallpox pustular eruption

and prostration, the 2 rises of temperature, and the centrifugal distribution of the rash. The presence of a recent successful vaccination renders a diagnosis of smallpox unlikely.

Hemorrhagic or toxic smallpox.

This variety is the severest form of the malady and is attended by the highest mortality. Curshmann (1875) has divided the condition into 2 types, the first named "purpura variolosa," also known as true hemorrhagic or black smallpox, and the second, which he has called "variola hemorrhagica pustulosa," is also referred to as vesicular hemorrhagic smallpox Ricketts and Byles (1908), however, pointed out that these 2 forms of toxic smallpox were not sharply differentiated

True hemorrhagic or black smallpox. This may commence with a prodromal rash, bright red in color. It may be accompanied by a high temperature and numerous hemorrhages into the skin of the groins, axillae, face, and other regions. Bleeding may also occur from any part of the body which is capable of hemorrhage (Ker, 1920) Death often occurs about the 3rd day before the true eruption has

had time to appear, but when it occurs on the 5th day, an early eruption may be evident.

"*Variola hemorrhagica pustulosa*" *or vesicular hemorrhagic smallpox.* The condition begins with high fever and may be preceded by a prodromal rash. Hemorrhages into the skin are a marked feature of the disease, and these may occur either before or after the appearance of the vesicular stage of the true eruption. The constitutional disturbance is usually very severe and the patient seldom lives long enough to reach the pustular stage.

Wilkinson (1943) in a full account of toxic smallpox in Asia, draws attention to the prostration, mental clearness, fetor, enlarged liver, rising respiration rate, and poorly developed focal lesions He found the "lobster" prodromal erythema to be followed invariably by a fatal course

Mortality

The death rate from smallpox varies according to the clinical severity of the disease. One of the most important papers dealing with the influence of the severity of the disease on mortality is that of Sweitzer and Ikeda'(1927). During the course of an epidemic of smallpox at Minneapolis, in 1924–5, affecting 581 persons, these authors reported the following death rates in the different clinical varieties observed Among 225 cases of the discrete type 14 died, so that the death rate was 6 *per cent. Among 151* confluent cases, 68 deaths occurred, the rate being accordingly 45 per cent Among 144 cases of the hemorrhagic type, 113 died, the mortality rate thus being 78 per cent. Purpuric smallpox (variola purpurica) was invariably fatal

The above figures offer a striking contrast between the death rates from the different forms of variola major, and alastrim, in which the disease is almost never a cause of death by itself (see Hay, 1938).

Clinical Pathology

The urine is diminished in volume and the specific gravity is high, it contains deposits of uric acid and urates, and occasionally albumin or sugar may be present The excretion of chlorides is reduced and sulfates increased, blood, when found, is regarded as a bad prognostic sign.

Blood changes. According to Magrath, Brinckerhoff, and Bancroft (1904), alterations occur in the total and relative numbers of the white blood corpuscles during an attack of smallpox. At the end of the incubation period lymphocytosis develops. This is followed by an absolute leukopenia at the time of onset of symptoms At the stage of pustulation when secondary pyogenic invasion occurs, polymorph leukocytosis takes place Other workers who have made observations on the blood changes are Ferguson (1902), Erlenmeyer and Jalkowski (1913), Schilling (1916), Simon (1918), and Hinojar and Corvacho (1940).

The blood changes in hemorrhagic smallpox have been investigated by Ikeda (1925) and Easton (1945) There is a moderate leukocytosis involving granulocytes or lymphocytes, a marked and progressive thrombopenia develops, accompanied by the appearance of polychromasia, punctate basophilia, and normoblast cells. Myelocytes, metamyelocytes, Turck cells, and neutrophil leukocytes showing nuclear degeneration are also found, but the erythrocyte count and hemoglobin percentage remain normal.

Inflammatory Complications of Smallpox

In cases exhibiting severe secondary infection of the skin, boils, abscesses, carbuncles, erysipelas, or permanent loss of hair may result. Eye lesions, such as keratitis, iridocyclitis, and hypopyon ulcers may occur. Smallpox is one of the commonest causes of blindness in India (Sen, 1945). Otitis media, osteomyelitis, cystitis, orchitis, and oophoritis have also been observed

Nervous Complications

Cases of smallpox complicated by involvement of the central nervous system have been reported by numerous workers (Leroy d'Etiolles, 1856, Gubler, 1860, Bernhardt, 1871; Gubler and Laborde, 1871; Roger and Damaschino, 1871; Westphal, 1872, 1874; Wohlrab, 1872, Whipham and Myers, 1886; Combemale, 1892; Spiller, 1903; Aldrich, 1904; Kleineberger, 1913, Wilson and Ford, 1927; *Report*, 1930, Troup and Hurst, 1930, Marsden and Hurst, 1932; Brouwer, de Jongh, and Rochat, *1933, Rice and Carey, 1933, Marsden, 1934). The literature of the subject* has been reviewed by various writers (in particular Aldrich, Turnbull and McIntosh, 1926, Wilson and Ford, Marsden and Hurst).

These complications are rare, and usually develop 5 to 13 days after the appearance of the focal rash, various clinical features may occur, such as encephalitis, speech disorders, delirium, meningitis, spinal symptoms, and peripheral neuritis.

The histopathology has been described by various recent workers who have found that it is, as a general rule, indistinguishable from that of postvaccinal encephalitis (see Ch. XXXV)—demyelination and perivascular infiltrations being characteristic (Wilson and Ford, 1927; *Report*, 1930, Troup and Hurst, 1930, Marsden and Hurst, 1932).

Pathology of Variola Major

The skin.

The appearances presented depend, of course, on the stage of the disease at which the patient dies, but this usually occurs from the 10th to the 13th day, at which time the skin eruption has reached the pustular stage. The pocks are mostly superficially situated

The histology of the smallpox skin lesion has been described in great detail by Michelson and Ikeda (1927), who found that the earliest signs of infection are slight edema of the papillary body, and plasma cell infiltration around the capillaries and blood vessels of the affected site, accompanied by small hemorrhages at the tips of the papillae. Other changes are perivascular lymphocytic infiltration, separation of the deepest epithelial cells, and the formation of areas of "ballooning" and reticular degeneration in the more superficial epithelium

As the condition proceeds, a clear serous exudate develops, which fills the spaces formed within the degenerated epithelial layer so that gradually a vesicle, consisting of an accumulation of fluid inside a number of spaces formed by strands of reticulum, results In its structure, therefore, the vesicle is a multiloculated lesion which cannot be collap ... , the vesicle fluid becomes infected with ... as staphylococci, pneumococci, or hemo ... 922) This is followed by polymorphonuclear leukocytic infiltration, the formation of pus, rupture of the reticulum strands, and the development of an umbilicated pustule.

Councilman, Magrath, and Brinckerhoff (1904) state that these changes may either occur wholly within the epidermis, the fluid contents of the pock being separated from the corium by a layer of comparatively intact cells, or the corium may constitute the bottom of the pustule, in which case there is usually necrosis of the papillary border

Varying degrees of hemorrhage may accompany the changes already mentioned, thus, Ikeda *(1925)* and Michelson and Ikeda *(1927)* have distinguished 3 varieties of the hemorrhagic form of the disease according to the histological changes present, which are as follows

1. The hemorrhagic pustular form, this is characterized clinically by the presence of hemorrhages in or under fully developed pustules.

2. The primary purpuric type, this appears clinically as an intense cherry-colored erysipeloid exanthem showing marked subcuticular and submucous ex-

travasations of blood, histologically, there are no variolous changes in the epithelium overlying the purpuric spaces

3 This variety has been named the secondary purpuric form. Clinically it includes those cases which develop purpuric signs after the development of the pocks, the principal histological features are typical vesicular and pustular lesions of the epidermis accompanied by hemorrhages into the corium, as in the case of the primary type.

Elementary bodies are present in the vesicle fluid during the early stage of the disease The inclusion bodies occur within the cytoplasm of epithelial cells, they are usually acidophilic, and surrounded by an unstained halo.

Healing of the pock is brought about by absorption of the fluid, and superficial necrotic changes, eventually a crust develops consisting of a mass of degenerated epithelial cells, leukocytes, and debris enclosed between two layers of horny epidermis. The crust is eventually cast off.

The respiratory system.

The mucous membrane of the palate, fauces, pharynx, larynx, and trachea is swollen and edematous, and may present numerous discrete nodular elevations (or pocks), with areas of necrosis and submucous hemorrhage (see Ponfick, 1872, Eppinger, 1876, Breynaert, 1881, Huguenin, 1897, Roger, 1902, and Perkins and Pay, 1903)

In severe cases the lungs may be edematous, swollen, and hemorrhagic, with *patches of atelectasis, areas of bronchopneumonic consolidation, and occasionally* gangrene. Gelatinous pleurisy and hypostatic pneumonia have been described in cases of purpuric smallpox by Sorensen and Sorensen (1925). Desquamation of bronchial epithelium with hemorrhages into the peribronchial tissues was observed by Ikeda (1928), Keysselitz and Mayer (1909) found Guarnieri bodies in alveolar cells.

Other systems.

Alimentary tract. The lesions are similar to those described in other organs, e g, hemorrhages with edema, and may occur in the esophagus, the lining of the stomach, and the intestines.

Liver. Cloudy swelling, fatty degeneration, and marked hemorrhages beneath the capsule and into the parenchyma have been described (see Roger, 1902, Keysselitz and Mayer, 1909, Ikeda, 1928, and Lillie, 1930)

Kidneys Cloudy swelling, degeneration of the convoluted tubules, petechial [illegible]

[illegible]

absence of polymorphonuclear leukocytes, decrease in the number of myelocytes, and dilatation of the rib marrow

Spleen This is usually normal but may show septic infarcts

Heart This structure shows cloudy swelling of the myocardium with punctate hemorrhages into the epicardium Bacterial endocarditis may be found if secondary *infection has been responsible for death*

Thymus Multiple focal hemorrhages and necroses are present The reticulum [illegible] and replaced

[illegible] ltrations have [illegible] ation by Per-[illegible]

Other organs. The testicles and ovaries may show small lesions (Béraud, 1859, Chiari, 1886, 1889) Similar changes may be evident in the epididymis and prostate.

The brain appears congested, and the blood vessels of the pia mater are injected The placenta shows areas of degeneration and polymorphonuclear infiltration.

ALASTRIM

This disease is also known as variola minor, amaas, kaffir-pox or milk-pox

Clinical Features

After an incubation period of 10 to 15 days, the disease is ushered in with a sudden rise of temperature to 103° to 104° F. or higher, accompanied by headache, backache, pains in the limbs, vomiting, furring of the tongue, and constipation. On the 2nd to the 5th day of the patient's illness a rash develops, and at the same time the constitutional symptoms abate in severity, while the temperature falls (McSweeney, 1930–1). Vesicles next develop, and simultaneously there may be a feeling of tension beneath the skin, but the severe constitutional upset, mental depression, and the characteristic odor of the skin often observed at the corresponding stage during an attack of variola major are absent in alastrim

The eruption does not appear in crops, but takes at least 3 days to develop. It begins on the face, forehead, dorsum of the wrists and the forearms, and in severe cases the whole body may become covered. The scalp, mouth, hard and soft palate, the cheeks, trachea, larynx, palms of the hands, soles of the feet, and genitalia are usually involved to a lesser extent; the neck, upper part of the trunk and abdomen, the inner side of the thigh, and the circumorbital area may escape attack (Moody, 1922). McSweeney (1930–1) states that in general, the lesions tend to be centrifugal in their disposition, and when present on the trunk, more pocks are found on the back than on the chest and abdomen (see also Rudolph, 1911). They also show a tendency to avoid the midline of the body and the axillary spaces. The lesions begin as a series of papules which 36 hours later turn into vesicles, measuring 4 to 5 mm in diameter, they are either dome-shaped or flat-topped with a dark-colored central area, and are filled with a clear exudate. On the 3rd day of the illness, this clear vesicular fluid becomes invaded by polymorphonuclear leukocytes, followed by infection with pyogenic organisms on the 6th day, after which a second slight rise of temperature may occur At this stage the skin lesion is a pustule, and is surrounded by a red areolar margin with an area of induration and subcutaneous swelling, so that, to the touch, it seems tense, hard, and shotty. Primary umbilication is absent, and on the 8th to 9th day absorption of the fluid occurs and secondary umbilication develops.

At this stage of the illness the skin lesions may either be delicate and closely set, or else coarse and discrete, alternatively, they may be confluent and hemorrhagic. On the 12th day, in a mild case of alastrim, convalescence is well advanced and desquamation should be completed by the 21st day. Elementary bodies occur in large numbers within the pock material Inclusions can be found in the epithelial cells

Occasionally the differential diagnosis of alastrim and variola major may present difficulties, and some of these have been referred to by Pierce (1935), van Campenhout (1935) Marsden (1936) and Hay (1938) give full accounts of the clinical features.

Complications

Bronchopneumonia is the most serious complication, and when lesions develop on the surfaces of the respiratory passages, septic material tends to accumulate and obstruct breathing Impetigo, boils, and eczema are other sequelae.

Pathology

If death occurs, it usually takes place after about 10 to 12 days, the most fatal cases being those with confluent or hemorrhagic rashes.

The skin.

We shall limit our description of the histology of alastrim to quoting the work of Torres (1935–6), who has devoted a considerable amount of study to this matter. The following description is based, he states, partly on human biopsy tissue and partly on material obtained from infected monkeys There are 4 main stages in the development of the alastrim lesion (*a*) proliferation, (*b*) vesiculation, (*c*) pustulation, and (*d*) desquamation

(*a*) *Proliferation* The epidermis is thickened, and cellular proliferation occurs both upward and downward as a definite hypercanthosis; mitoses are not conspicuous Numerous malpighian cells contain the characteristic alastrim basophilic intracytoplasmic bodies (see p. 340) The nuclei of malpighian cells are swollen, and contain small vacuoles. There is marked inflammatory change in the papillary bodies, and the corium below the hypertrophied epidermis is found to be compressed The papillary eminences are narrowed and obliterated. The papillary lymph vessels are dilated, and vessels are surrounded by numbers of polymorphs and endothelial cells Torres stresses this change as supporting the view that the primary reaction in alastrim is a vascular change in the dermis, an initial anemia being followed by hyperemia and edema Later there occurs degeneration in the malpighian cells.

(*b*) *Vesiculation* In this stage multilocular vesicles form, each vesicle occurs in the top of the hypertrophied malpighian layer. Compared with variola major, "balloon" degeneration is rather scarce, but is found also in the upper layers of the stratum germinativum, associated with reticulating reaction This reticulating degeneration is the main change in the vesicles of alastrim, and is evidenced by the formation of minute vacuoles in the cytoplasm of many of the malpighian cells, which eventually become confluent with the formation of a multilocular vesicle The malpighian cells of the bottom and sides of the vesicle are quite normal in appearance, in variola, however, these cells show "balloon" degeneration or necrosis The most typical feature is the presence of giant epithelial cells at the bottom and sides of the vesicle, they may also occur in the middle layer of the hypertrophied epidermis The vesicle is roofed by the horny layer and compressed superficial cells of the stratum germinativum

(*c*) *Pustulation.* Polymorphs begin to accumulate during the stage of vesiculation, but there is no abrupt onset of the stage of pustulation When the pustule is well advanced the vesicle is packed with polymorphs, there is much necrosis of the malpighian cells

Inflammatory changes are marked in the cutis in both the vesicular and pustular stages Cytoplasmic inclusions are regularly found in malpighian cells at the bottom and sides of the vesicle In human beings intranuclear bodies are very rare.

(*d*) *Desquamation.* The lesion heals by the formation of a crust composed of devitalized cells and degenerate polymorphs, this crust is eventually separated by the ingrowth of epithelium from the sides

Other organs.

The respiratory passages may contain septic and necrotic material, and pocks may be evident throughout the entire length of the trachea The lungs may show evidence of hemorrhagic bronchopneumonia. Other changes observed are fatty degeneration with hemorrhages into the kidney, hemorrhages into the epicardium at the root of the aorta, the walls of the le[illegible]

The stomach contains altered blood, [illegible]
its mucous membrane The intestines ar[illegible]
may be occluded with necrotic debris, [illegible]
being the principal portions affected The mucous membrane lining the bladder shows similar changes The liver shows fatty degeneration, but the spleen usually appears unaffected (see Moody, 1922).

The brain appears congested, and the blood vessels of the pia mater are injected. The placenta shows areas of degeneration and polymorphonuclear infiltration.

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If death occurs, it usually takes place after about 10 to 12 days, the most fatal cases being those with confluent or hemorrhagic rashes.

LABORATORY DIAGNOSIS OF SMALLPOX

Laboratory diagnosis is of the greatest assistance in confirming the clinical diagnosis, particularly in the initial cases of an epidemic. Laboratory methods may prove of value in cases doubtful on clinical grounds. The diagnostic methods have improved very considerably of recent years, and full accounts based on extensive personal experience have been given by Downie (1946, 1947) and van Rooyen (1946).

Collection of Specimens, and Selection of Test

van Rooyen (1946) recommends the following procedures, the actual technique of the tests being described later (Table 11 summarizes the recommendations) It should be remembered that a retrospective diagnosis may be made in cases where laboratory examinations are not immediately available, for variola virus survives in crusts in the refrigerator, or even at room temperature for over a year (North, Broben, and Mengoni, 1944, Downie and Dumbell, 1947 *a*, *b*)

Day I Initial febrile stage prior to rash

There is no laboratory procedure which will provide useful information

Days II and III Bathing-drawers rash and maculopapular eruption

If the groins and buttocks show an early petechial rash, the diagnosis can be confirmed beyond doubt in less than half an hour by examining lesions for the presence of elementary bodies The specimen is collected thus A sterile sharp-pointed scalpel and three well-cleaned glass microscopic slides are required, with a bottle of ether and absolute alcohol Choose a dozen typical petechiae, clean them with cotton wool soaked in ether, scrape the red central base of each lesion with the point of the knife, and rub the exudate with a circular rotary motion over the surface of the slide Four smears are made on each slide. Thereafter the preparations are allowed to dry in air for 10 minutes, treated with sterile physiological (0 86 per cent) saline for 3 minutes, placed upright in a rack, allowed to dry, flooded with a mixture of equal volumes of alcohol and ether for 3 minutes, the mixture is poured off, the slides are dried in air, wrapped in clean paper and sent to the laboratory with the request that they be stained by Paschen's method, and examined for presence of variola-vaccinia elementary bodies The bacteriologist should be able to provide a reply within 30 minutes after receipt of the specimen. If virus bodies are found, the case may safely be regarded as variola, but if no elementary bodies are visible, scrapings should be repeated every 4 hours

Paul's test provides a method of isolating virus, and if the result is positive the diag-

ts, but

Days IV and V Vesiculation

goc

cap

the complement fixation or flocculation reactions Both procedures occupy about 12-24 hours, and are highly specific for the variola-vaccinia group

Days VI to VIII Pustulation

Pustulation is generally pronounced at this stage, and the introduction of streptococci, staphylococci, *B coli*, and other secondary contaminating organisms makes it difficult to carry out the direct film technique By carefully selecting lesions containing clear fluid, scraping the red periphery of individual pocks, and by avoiding the presence of serosanguineous pus, it is occasionally possible to obtain reasonably satisfactory preparations showing elementary bodies Paul's test can be carried out, or virus recovered by intratesticular inoculation of rabbits with pustule contents Antiseptics must be used to suppress the growth of bacteria and cocci Penicillin must also be used when inoculating eggs

Serological tests offer the best prospect of arriving at a laboratory diagnosis after the

SMALLPOX MODIFIED BY IMMUNITY

Variola major and minor may occur in modified form in persons protected by vaccination. The patients usually have little or no constitutional upset or perhaps a mild influenzal type of illness. There may be headache, fever, and prostration There are seldom over a dozen focal lesions, and these are often on the wrists, forehead, and backs of the hands. Of recent years many such cases have been noted, and they have often given rise to outbreaks of unmodified smallpox (D'Arcy, Moore, and Whetter, 1943; Beaumont, 1946, Conybeare, 1946).

As lately restated by Wilkinson (1942) smallpox may be modified by vaccination in various ways (1) The toxic phase is attenuated. (2) The total number of skin lesions is lessened. (3) The natural maturation of these lesions is shortened These 3 effects disappear, as immunity resulting from vaccination wanes, at different rates. First to be lost is the toxemia-modifying property, then the property of modifying the number of lesions, and lastly the property of altering maturation. Accordingly, toxic smallpox not uncommonly kills those with good vaccination scars. In others, a severe toxic phase may be followed by a focal phase of a few lesions only.

It is probable that modified smallpox may very rarely occur in persons showing some natural immunity, or associated with the invasion of a strain of virus of modified virulence.

It may be difficult to make the diagnosis between varicella and modified variola, but the following points are helpful (League of Nations, 1940) (1) In varicella the rash may precede the fever or appear with it. (2) The varicella lesions are more superficial. (3) The maturation is more rapid in varicella, and crops occur at all stages (4) In varicella the distribution of the lesions favors the less exposed parts

Contacts of smallpox, particularly those protected by vaccination, may develop a febrile illness without rash, and this has been known as "contact fever," abortive smallpox, or variola sine variolis (Conybeare, 1939, 1946, Bowe, 1942; Napier and Insh, 1942, Easton, 1945, Boul and Corfield, 1946). This type of illness may be a cause of confusion in the diagnosis of smallpox, for example in reception houses, and in many cases it is probable that the symptoms are due to a recent vaccination and not to variola, or alternatively are nonspecific (Tyrrell, 1942) The symptoms, those of a toxemia, include headache, fever, backache, malaise, shivering, and catarrh, but rapidly clear up Howat and Arnott (1944) described fine diffuse mottling in x-ray pictures of the lungs, presumably due to a virus pneumonia

SPECIFIC TREATMENT OF SMALLPOX

Sulfonamides have been widely used, particularly in variola major (Hinojar and Corvacho, 1940, Patel and Naidu, 1940, Couzi and Kircher, 1941, Vengsarkar, Poonen, and Walavalkar, 1942, Wilkinson, 1942, Rao and Natarajan, 1943, Cottrell and Knights, 1943, Leishman, 1944, Sen Gupta, 1944, Vengsarkar and Rangnekar, 1944, Easton, 1945, Osborne, 1945) The majority has recorded definite benefit in the following respects, especially with sulfathiazole administered early in the disease, the pustular stage is less pronounced, secondary invasion of the pocks is less marked, and secondary fever may be abolished, complications are less frequent; convalescence is shortened, and mortality is reduced Toxemia is not affected.

Convalescent serum, from recently vaccinated persons or smallpox convalescents, has been used, and it has been claimed that the course is shortened and the pustular stage aborted (Couzi and Kircher, 1941, Panja and Das, 1942, Wilkinson, 1943)

Antibiotics Some favorable reports on the use of penicillin have appeared (Jeans, Jeffrey, and Gunders, 1944, Foulis, 1945, Weeks and McClelland, 1947)

pustules be collected on glass slides, and allowed to dry. On reception in the laboratory, the exudate was dissolved in saline and applied to the scarified cornea of a rabbit. The animal was killed after 48 to 72 hours, the eye removed and placed in corrosive sublimate Examination with a hand lens showed the presence of raised white papular opacities in positive cases It should be noted that many years earlier, Guarnieri (1892) and Huckel (1898) had shown that lesions developed on the rabbit's cornea after inoculation with the contents of smallpox vesicles, to Paul, however, goes the credit for introducing this reaction as a diagnostic test. The value of Paul's test has been confirmed by various authors (Gins, 1916 *a*, *b*, 1930 *a*, Gasteiger and Niederwieser, 1931–2, Jadassohn *et al*, 1931; Torres, 1935–6, Torres and Teixeria, 1938)

We shall now describe in some detail the method of applying Paul's test as recommended by Scott and Simon (1923).

Collection of material. The vesicles or pustules are wiped over with alcohol, and then incised with a sterile lancet. The contents are collected on slides, and sent to the laboratory. Scabs or crusts may also be used

The test proper. The rabbit's corneae are scarified with a dissecting needle, the material on the slide is emulsified in saline, and transferred to the corneae on a coverslip or platinum loop. After 48 hours, the corneae are examined with a hand lens, and in positive cases tiny lesions like air bubbles are seen, perhaps with depressed centers The rabbit is then killed and the eyes placed in a sublimate bath (2 volumes of a saturated solution of mercuric chloride, and 1 volume of 96 per cent alcohol). After 2 to 4 minutes the eyes are transferred to 70 per cent alcohol Examination should then be carried out with a lens, against a black background. In positive cases the background of the cornea appears white, but there are a number of intensely white opaque elevations, sometimes with a central crater, these lesions vary in number from 3 to 24, and although usually discrete, may be confluent.

Rapid histological examination This is carried out as follows

(*a*) Place the whole eye in sublimate, and then in 70 per cent. alcohol.
(*b*) Then place in sublimate again for 10 minutes
(*c*) Excise the cornea and place in absolute alcohol for 1 minute
(*d*) Place the cornea in each of the following for 1 minute iodized alcohol, absolute alcohol, equal parts of alcohol and chloroform, chloroform, saturated solution of hard paraffin in chloroform at 40° C, liquefied hard paraffin at 60° C.
(*e*) The cornea is then embedded, and sagittal sections are cut.

Microscopical examination of the cornea Microscopically, there is to be seen a number of epithelial hummocks, at first, these elevations are due to a swelling of the basal epithelial cells which throws the superficial cells out of their normal alinement The epithelial layer appears twice as broad as usual The changes are most pronounced in the center of the elevation, where necrosis occurs Guarnieri bodies can be found in the lesions

Paul's test in alastrim Torres (1935–6) reported as follows on the result of Paul's test in alastrim When placed in the sublimate bath, in certain cases tiny yellowish-gray elevations can be seen along the lines of scarification. In other cases the only naked-eye change is a uniform thickening along some of the scarifications In th[illegible]
cen[illegible]

as i[illegible]
rest[illegible]

Downie (1946) has not found the test reliable in Britain in cases of variola major.

5th day of disease As much pustular exudate as procurable should be gathered into glass capillary tubes, sealed with a flame and sent to the laboratory.

Days IX to XIV: Desiccation and desquamation.

During the process of drying of the pustules followed by desquamation, serological tests are the quickest method of securing laboratory evidence of infection. They are suitable in missed cases of smallpox seen for the first time at a late stage of the disease From the patient and his garments crusts and scabs should be removed with forceps, and placed in a dry, sterile, glass phial. All the available material should be collected An attempt to isolate virus should also be made, since crusts retain their infectivity for long periods of time

TABLE 11

LABORATORY DIAGNOSTIC PROCEDURES IN SMALLPOX

Day of Illness	*Clinical Condition*	*Material Available and Specimen Required*	*Test to be Performed*	*Duration of Laboratory Examination*
1	Fever, headache	Nil	Nil	Nil
2	Petechial "bathing drawers" or other prodromal rash	Scrapings from skin petechiae on slides	Microscopic examination for Paschen elementary bodies, electron microscopy	30 minutes
		In 1 c c of saline	Paul's test	2 3 days
			Isolation of virus in eggs and animals	4-10 days
3	Maculopapular eruption	as above	as above	as above
4 to 5	Vesicles	If vesicles are small, skin scrapings from base of 6 or more lesions to be made on glass slides.	Microscopic examination for Paschen bodies	30 minutes
		If vesicles are large and contain much fluid, fill capillary tubes	Complement fixation reaction	12–24 hours
		Vesicle fluid in capillary tubes	Paul's test	2–3 days
			Isolation of virus in eggs and animals	4–10 days
6 to 8	Pustules	Exudate from pustules in sterile screw-cap phial	Complement fixation tests	12–24 hours
			Flocculation test	12–24 hours
			Paul's test	2–3 days
			Isolation of virus in eggs and animals	4–10 days
9 to 14	Scabs	Desquamated epithelium to be gathered in a dry, sterile screw-cap phial	as above	as above

Technique of Tests

Examination for elementary bodies.

Films are stained by Paschen's, Gutstein's, Morosow's or other method (see Ch III). Microscopical examination shows very large numbers of small apparently spherical granules measuring 0 2 μ Granules of similar appearance and numbers would be found in a vaccinial lesion, but not in varicella van Rooyen and Illingworth (1944) used this method with considerable success in the Middle East campaign (see also Illingworth and Oliver, 1944) Downie (1946) also reports favorably. The test is applicable particularly to the early stages, before pustulation.

When facilities are available, electron microscopy affords a quick and accurate method of detecting the EB's of the variola-vaccinia group (Nagler and Rake, 1948, and personal observations)

Paul's test.

In 1916 (*a, b*) Paul introduced a laboratory test for the diagnosis of smallpox (see also, 1917 *a, b*, 1918, 1919) He recommended that the contents of variola

MILKER'S NODES

The condition, as its name implies, is acquired by milking *infected* cows, and, according to Bonnevie (1937), is characterized by the following clinical features The lesions usually develop on the hands and may commence as flat red papules (accompanied by pruritus) which in a week's time reach the size of a pea- or hazel-nut, appearing as well-defined hemispherical, reddish-blue, firm, elastic, and slightly tender nodules Suppuration next takes place, the fluid within then becomes absorbed, and the lesion flattens and disappears in a few weeks, but secondary infection and scar formation may develop in the more chronic forms of the illness
... any general disturbance of the patient's health, has been observed Cases have also been de-

The histological appearances of milker's nodes in certain respects resemble those seen in vaccinia, with the essential difference that in milker's nodes marked parakeratosis prevents the vesicles from rupturing

The etiology of the condition is obscure, but, according to Schultze *et al* (1927) and Stark *et al* (1934), the condition is due to infection with cowpox virus (see also Davies, Janes, and Downie, 1938, Tappeiner, 1938)

VACCINIAL INFECTION IN LABORATORY WORKERS

Workers in lymph institutes might be expected to be highly resistant to vaccinia Nevertheless vaccinal lesions may occur on the fingers and hands of persons shown to be highly immune by dermal tests (Broben and North, 1943; Horgan and Haseeb, 1944 *a*) These lesions clinically resemble milker's nodes or cowpox

KAPOSI'S VARICELLIFORM ERUPTION

Considerable interest has been taken in this condition, and a number of cases reported (e g , Juliusberg, 1898, Brown, 1934, McLachlan, 1934, Corson and Ludy, 1935, Goeckerman and Wilhelm, 1935).

It seems that at least 3 distinct disease entities are described under this title. In one group no virus can be isolated In the second group the etiological agent appears to be a member of the variola-vaccinia group, for Guarnieri bodies may be seen in the skin, and a virus with vaccinia-like properties may be isolated by suitable tests, such cases tend to occur in contacts of variola or vaccinia (Freund, 1934, Pepple, Murrell, and Fowlkes, 1942, Ronchese, 1943). The condition known as eczema vaccinatum may be identical with Kaposi's eruption

During the Glasgow 1942 outbreak of variola, 10 cases were seen, which, considering the rarity of the condition, argued for a connection with the variola-vaccinia group, the crusts of 2 cases gave positive serological tests (McKenzie, 1943).

In the third group, the herpes febrilis virus may be isolated from the lesions, and neutralizing antibodies develop in convalescence (Blattner, Heys, and Harrison, 1944, 1945, Lane and Herrold, 1944, Wenner, 1944, Lynch, 1945, Jaquette, Convey, and Pillsbury, 1946, Barker and Hallinger, 1947, Kipping and Downie, 1948).

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EPIDEMIOLOGY OF TRUE SMALLPOX AND ALASTRIM

Recent Geographical Distribution

In *Great Britain* in recent times, alastrim was prevalent in England and Wales, and in 1927 there were 14,767 cases, in 1935 there were no cases (see *Memorandum*

Inoculation of animals.

An attempt may be made, by inoculation of monkeys, to isolate the virus of variola from the vesicular or pustular contents. Torres and Teixeria (1934 a 1937), for example, have specially recommended inoculation of the skin of *M. mulatta* in the diagnosis of alastrim After a few days, histological preparations are made from the lesions and search is made for the characteristic inclusions. Similar methods may be applied to the diagnosis of variola major. It may be worth while inoculating rabbits dermally (see Ch. XXVIII).

Inoculation of eggs.

Of recent years, a number of authors has shown that the chorio-allantois is highly sensitive to the presence of variola virus, the membrane is inoculated with a suspension of material obtained from the skin lesion, after 3–4 days characteristic pocks develop, and EB's and inclusions can readily be found (for references see p. 346). The use of antibiotics may facilitate virus isolation.

Serological tests.

Of recent years the technique of the variola flocculation and complement fixation tests has been brought to a high level of perfection. By the application of these tests (fully described on p. 370), it is possible definitely to establish the variolar nature of scabs from suspected cases. These results unfortunately do not distinguish between vaccinia and variola.

Antibodies develop in convalescence, and may enable a retrospective diagnosis to be made. These antibodies develop, of course, also after vaccination, and this may complicate diagnosis

HUMAN COWPOX

Human cowpox is a relatively mild disease (in comparison with variola major or minor), which affects only dairymaids and others who handle the udders and teats of infected cows Owing to its very limited distribution, human cowpox presents no epidemiological problems from the public health administrative point of view Cowpox, nevertheless, is of immunological importance in view of the protection which it affords against variola, as originally shown by Jenner

Among the more recent clinical descriptions of cowpox in man, Cathie (1932), for example, has reported a case occurring in a previously vaccinated farmer's wife who acquired the disease as the result of handling an infected milch cow The earliest lesion was a small red spot about the size of a pin's head which appeared on the patient's fingers, and developed into a blister filled with clear fluid that gradually enlarged in size to about 1 centimeter in diameter. At this stage of the lesion the patient complained of pruritus, general malaise, swelling of the hands and elbows, and axillary adenitis Pustules were also present on the anterior and posterior aspects of the upper arms and calves of the legs, and a temperature reaching 100° F. was recorded Healing occurred quickly, and on the 7th day the majority of the lesions had dried up

Taylor (1932) mentions 2 cases of cowpox acquired by farm laborers, who had contracted the disease through milking an infected cow which had blisters on its udder and teats In one of these patients the lesions were distributed on the forearms, and in the other they were present on the face, it was of interest to note that both persons had been vaccinated some 15 years previously (see also Kaiser and Weinfurter, 1932, and Davies, Janes, and Downie, 1938, for other accounts)

Kaiser and Gherardini (1933–4) reported the development of firm red nodules on the fingers, hands, and face in addition to the usual vesicular lesions of cowpox

Vaccinia in man may be transmitted mechanically to cows, spread in them, and reinfect man, the clinical picture closely resembling natural cowpox (Gray, 1943)

MILKER'S NODES

The condition, as its name implies, is acquired by milking infected cows, and, according to Bonnevie (1937), is characterized by the following clinical features The lesions usually develop on the hands and may commence as flat red papules (accompanied by pruritus) which in a week's time reach the size of a pea- or hazel-nut, appearing as well-defined hemispherical, reddish-blue, firm, elastic, and slightly tender nodules Suppuration next takes place, the fluid within then becomes absorbed, and the lesion flattens and disappears in a few weeks, but secondary infection and scar formation may develop in the more chronic forms of the illness The condition does not give rise to any general disturbance of the patient's health, although occasionally lymphangitis has been observed Cases have also been described by Becker (1940)

The histological appearances of milker's nodes in certain respects resemble those seen in vaccinia, with the essential difference that in milker's nodes marked parakeratosis prevents the vesicles from rupturing

The etiology of the condition is obscure, but, according to Schultze *et al.* (1927) and Stark *et al* (1934), the condition is due to infection with cowpox virus (see also Davies, Janes, and Downie, 1938, Tappeiner, 1938).

VACCINIAL INFECTION IN LABORATORY WORKERS

Workers in lymph institutes might be expected to be highly resistant to vaccinia Nevertheless vaccinial lesions may occur on the fingers and hands of persons shown to be highly immune by dermal tests (Brohen and North, 1943, Horgan and Haseeb, 1944 *a*) These lesions clinically resemble milker's nodes or cowpox.

KAPOSI'S VARICELLIFORM ERUPTION

Considerable interest has been taken in this condition, and a number of cases reported (e g, Juliusberg, 1898, Brown, 1934, McLachlan, 1934, Corson and Ludy, 1935, Goeckerman and Wilhelm, 1935).

It seems that at least 3 distinct disease entities are described under this title. In one group no virus can be isolated In the second group the etiological agent appears to be a member of the variola-vaccinia group, for Guarnieri bodies may be seen in the skin, and a virus with vaccinia-like properties may be isolated by suitable tests, such cases tend to occur in contacts of variola or vaccinia (Freund, 1934, Pepple, Murrell, and Fowlkes, 1942, Ronchese, 1943) The condition known as eczema vaccinatum may be identical with Kaposi's eruption

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EPIDEMIOLOGY OF TRUE SMALLPOX AND ALASTRIM

Recent Geographical Distribution

In *Great Britain* in recent times, alastrim was prevalent in England and Wales, and in 1927 there were 14,767 cases, in 1935 there were no cases (see *Memorandum*

on Smallpox, 1938). *Until recently, variola major has been rare, but "imported"* smallpox occurred in 1929 in Glasgow, and in Blackburn in 1934. During and since the Second World War there have been numerous importations of variola major into the British Isles. *The main recorded outbreaks are as follows:*

1942 Glasgow (Macgregor, 1942, Macgregor and Peters, 1942);
Edinburgh (Clark, 1944);
Fife (Fyfe and Fleming, 1943; Health Bulletin, 1943); Sutherland (1943) discussed the epidemiology of these outbreaks

1944, a hospital in Middlesex (Bradley, Davies, and Durante, 1946).

1946, Essex (Boul and Corfield, 1946); Merseyside (Peirce, 1947; Srallybrass, 1947); and more generalized, there being a total of 15 separate importations (Maddock and Conybeare, 1946; Bradley, 1947, Lancet, 1947).

These outbreaks were usually attributed to one or other of the following sources:

1. A case, often misdiagnosed, arriving by ship from India or the Middle East, and giving rise to infection in other passengers or crew.
2. A misdiagnosed case in a general hospital.
3. Infection from highly vaccinated persons, recently returned from abroad, developing modified smallpox.
4. Arrival by air of passengers in the incubation period of smallpox (see *Lancet*, 1946, Smith, 1948).

In *North America* the mild variety of smallpox is the prevailing clinical type (see Chapin and Smith, 1932, Dauer, 1946). Smallpox was very rare between 1932–

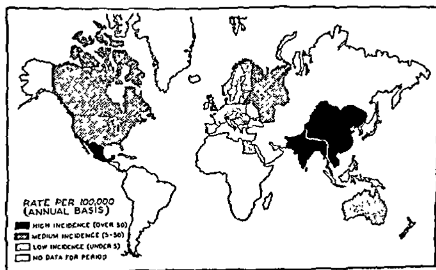


FIG 27 The world incidence of smallpox and alastrim (Hedrich, 1936). (Reproduced by courtesy of the Director of Public Documents, Washington)

1946, but recently there have been outbreaks of variola major (see Palmquist, 1947, Weinstein, 1947).

In many parts of the *Middle East and Africa*, both forms of smallpox are endemic, and frequently epidemic In the Second World War, a number of cases occurred in service personnel (van Rooyen and Illingworth, 1944; Illingworth and Oliver, 1944, Norris, 1944, Easton, 1945)

British India is the principal endemic focus of Asia There is a maximal inci-

dence in April. There is a rough 7-year cycle, peaks having occurred in 1927 and 1934 (see League of Nations, 1940).

Hedrich (1936) published the map illustrating the world incidence of the disease shown in Fig 27 and the information in Table 12.

Valuable information on the recent world incidence of smallpox has been published by the League of Nations (1934, 1943, see Fig 27), by the United Nations (1946), and by UNRRA (1945).

Mode of Spread and Pathogenesis

The case of smallpox is highly infective to unvaccinated contacts The infective agent persists in the skin lesions till the final stages, so that the case remains infective for an unusually prolonged period Infection may be transmitted by touch, or by fomites contaminated with saliva or by the skin, e g, wearing-apparel, bedclothes, cutlery, and crockery.

Variola virus in dried crusts may survive for over a year (Downie and Dumbell, 1947 *a*). Therefore the sickroom may become infected, and the virus survive therein for a prolonged period Downie and Dumbell actually isolated virus from the sweepings of a ward floor near the bed of a case. Flies play a considerable part in the dissemination of infection in the tropics.

It is probable that more usually cases of smallpox give rise to infection in close contacts by means of infected droplets of oral and nasopharyngeal secretion The question of whether infection can be spread more remotely by the air has been

TABLE 12

MORBIDITY, MORTALITY, AND APPARENT CASE FATALITY FROM SMALLPOX IN VARIOUS COUNTRIES, FOR VARIOUS PERIODS, 1921–30

Geographic area	*Number of cases for years indicated*	*Reported annual case rate per 100,000 population*	*Number of deaths for years indicated*	*Reported annual death rate per 100,000 population*	*Case fatality (deaths per 100 reported cases)*	*Years included*
	(1)	(2)	(3)	(4)	(5)	(6)
Mexico [3]	([2])	[4] 163.0	96,526	69.9	([2])	1922–30
British India [5]	979,738	74.2	414,659	31.4	42.3	1926–30
United States [4]	381,890	40.4	3,483	37	[7] 9	1921–30
Canada [3]	15,000	21.6	140	20	[7] 9	1924–30
England and Wales	73,115	18.6	246	06	.3	1921–30
Soviet Russia [8]	209,715	[9] 18.2	([2])	([2])	([2])	1921–30
Switzerland	5,494	[9] 14.1	16	04	3	1921–30
Chosen	20,721	[9] 11.5	5,979	3.31	28.9	1921–30
Java and Madura	20,069	[9] 5.3	3,695	98	18.4	1921–30
Egypt	5,353	3.8	1,222	.88	22.8	1921–30
Yugoslavia	4,241	[9] 3.4	915	74	21.6	1921–30
Rumania	3,764	2.3	730	44	19.4	1921–30
Italy	6,532	1.9	1,516	.43	23.2	1921–29
Ceylon	808	1.9	111	.27	13.7	1922–30
Japan	8,075	1.4	1,454	25	18.0	1921–30
France	1,573	1.0	363	22	23.1	1925–28
The Netherlands	728	92	23	03	[7] 3.2	1921–30
Scotland	452	93	26	05	[7] 5.8	1921–30
Finland	137	44	14	05	10.2	1921–29
Philippine Islands	318	39	56	07	17.6	1924–30
Germany	976	.15	161	.02	16.5	1921–30
Bulgaria	74	.15	14	03	[10] 18.9	1921–30
Austria	39	07	8	01	[10] 20.4	1921–30
Denmark	32	.09	3	01	[10] 9.4	1921–30
Norway	5	.02	0	00	[10] 0.0	1921–30
Sweden	6	01	4	09	[10] 66.7	1921–30

(Reproduced by permission of the Director of Public Documents, Washington)

much debated It is probable that droplet nuclei may be concerned (see the observations of Curshmann, 1875; Ker, 1920), and it has been a general belief that infection can be transferred over some distance, hence the siting of special smallpox hospitals. Millard (1944) has reviewed the evidence and suggests that it is stronger than often imagined, such a method of spread would explain the occurrence of cases without any apparent contact with a previous sufferer. Sabongy on the contrary (1923) does not believe that the virus can be conveyed through the air for any great distance, and cites the experience in the Government Fever Hospital, Cairo, where smallpox cases are nursed close to other compounds In Edinburgh in the 1942 epidemic, cases were nursed in a separate portion of the general fever hospital, but at no great distance from fully occupied wards, without any evidence of infection being conveyed

An important source of infection is the highly modified, perhaps ambulant, case of smallpox occurring in the immune person It is probable that such persons are the usual source of infections appearing without any evident contact with the fully developed disease. There is some evidence that healthy contacts may act as carriers (*Le Bourdellès, Lesaffre, and Rogez, 1946*).

There is a risk to undertakers dealing with corpses, and those engaged in disinfecting homes and fomites may contract the infection (Boul and Corfield, 1946)

It would appear that in the majority of cases, the infective agent enters the body by the nasal or buccal mucosa (see Blaxall, 1930).

Climatic Conditions and Smallpox

In India, Rogers (1926), by charting the average monthly smallpox mortality of 8 provinces together with the monthly rainfall, mean temperature, and both the relative and the absolute humidity or aqueous vapor tension (the last being the actual amount of moisture in the air measured by its pressure in terms of mercury, and essentially a measure of combined heat and moisture), has shown that the closest relationship exists between the last-named factor and the incidence of smallpox. *Thus the disease declined to its minimum with the rise of absolute humidity* to its maximum of 0 800 to 0.950

In England, Rogers (1928) has demonstrated the existence of a somewhat similar state of affairs during the years 1921 to 1927, when the mild type of smallpox was prevalent, he was able to correlate the case incidence with the prevailing climatic conditions Thus, a low absolute humidity favored the disease, and a high absolute humidity checked it He also pointed out that, in England, the mean temperature curve closely followed the absolute humidity curve and accordingly a cold winter and spring was a likely factor in the increase of cases.

The Control of Smallpox

The methods used in the control of smallpox, based on isolation and vaccination, have been admirably presented by Butterworth (1942), and the following account is partly based on his recommendations

Isolation.

The patient must be isolated, as infectivity persists till the lesions have healed It has been usual to isolate cases in special smallpox hospitals sited at a distance from other dwellings (For details regarding the planning, designing, and siting of [illegible] 1935, or other textbook on Public Health) [illegible] of variola major, it was shown that cases [illegible] the general fever hospital in cottages only 85 feet from fully occupied wards (Clark, 1944). The following precautions were taken All patients and staff in the hospital were vaccinated All admissions were vaccinated during the epidemic. The nursing staff were specially chosen, and also performed domestic duties. During the outbreak, they did not leave the grounds of

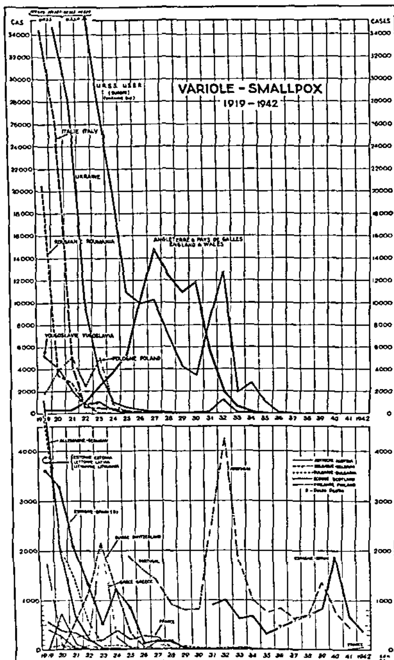


FIG 28 Chart showing incidence of smallpox in Europe, 1919-1942 (Reproduced from *Week Epidem. Rec*, 1943, *18*, 117, by courtesy of United Nations Health Organization)

the smallpox unit. Laundry was washed out in the unit and carbolized before going to the hospital laundry.

It must not be forgotten that ambulance drivers and similar staff must also be protected by vaccination.

It may be advisable to nurse smallpox patients on burnable mattresses, the excreta should be carbolized before disposal (see Conybeare, 1939, Gordon, 1939 *b*).

Disinfection.

The home of the case must be disinfected, e g, with formalin, and the bedding and clothing steam-treated. The personnel carrying out these tasks must be protected by recent vaccination.

Surveillance and vaccination of contacts.

A complete list of contacts must be made, including those less intimately exposed, such as tradesmen, and colleagues at the place of employment. These contacts should be vaccinated or revaccinated unless they have been done successfully very recently. Wherever possible they should be subjected to surveillance by officers of the Health Department, being visited daily for 3 weeks A medical officer should visit all contacts reported as showing illness. It may be feasible to take all intimate contacts into a reception house (see, e.g, Clark, 1944).

Common lodging houses and hostels.

These establishments should be visited during times of epidemic prevalence, as merchant seamen, vagrants, and similar persons are not infrequently concerned in the spread of smallpox

Schools.

Cases are of course excluded till pronounced free of infection Contacts are excluded for 21 days unless recently successfully vaccinated (Memorandum on *Closure of and Exclusion from School, 1942*). The head teacher should be instructed to send a daily list of absentees to the health department

Mass vaccination.[1]

As has been shown, the "sheet-anchor" of the control of smallpox is the application of vaccination to all contacts, close and remote. As long as an outbreak is of small dimensions, and contacts can be traced in each patient with a previous case, there is little necessity to embark on a mass vaccination campaign, with its attendant risks of postvaccinal encephalitis and other less serious complications. However, when the outbreak begins to spread, and when cases appear where no evident contact can be traced, it is usual to recommend the introduction of a mass vaccination campaign The spread of smallpox is capricious, and sometimes when the disease breaks out under conditions apparently very favorable for spread, this does not eventuate (Bradley, Davies, and Durante, 1946) The situation must therefore be carefully assessed before the decision to undertake a campaign of vaccination of the general public is taken.

For many years Millard has been opposed to a policy of general vaccination in a community as a means of checking the spread of smallpox in Britain (see 1943 *a*, *b*, 1945 *a*, *b*). He would concentrate on the vaccination of known contacts as the means of bringing an outbreak under control, and does not believe that there is much risk of the epidemic getting out of hand He feels that both the Edinburgh and Glasgow (1942) outbreaks were in fact already on the wane when mass vaccination was introduced

[1] The technique and principles of vaccination are discussed in Chapter XXXIV.

Seaport quarantine procedure.

In every seaport, laws exist for dealing with outbreaks of smallpox on ships entering that harbor Although individual regulations vary, in general they entail examination and compulsory vaccination of contacts, followed by a period of surveillance, *disinfection of clothing may be carried out* (Draper, 1925, Smith and Hart, 1925) Hutchison (1945), on the basis of his experience as a Port Medical Officer, questions the value of surveillance of contacts, which throws a heavy strain on the staff of health departments

...se to vaccina-

convenience,

action of immunity (+ to ++++). The Station does not accept a + reaction either for release or issue of certificate of "immunity." The reaction must be more definite (Hooker, 1925).

The International Sanitary Convention (1944) *laid down the following recommendations*

ARTICLE 42

Ships which have had a case of smallpox on board either during the voyage or at the *time of arrival may be subjected to the following measures*

1. Medical inspection.
2. The patients shall immediately be landed and isolated
3. Other persons reasonably suspected to have been exposed to infection on board, and who, in the opinion of the sanitary authority, are not sufficiently protected by recent vaccination, or by a previous attack of smallpox, may be subjected to vaccination or to observation or to surveillance, or to vaccination followed by observation or surveillance,

and which the sanitary authority regards as infected shall be disinfected

The ship shall immediately be given free pratique

For the purpose of this Article "recent vaccination" shall be taken as meaning evidence of successful vaccination not more than 3 years or less than 14 days previously, or evidence of an immune reaction.

It rests with each Government to take after disembarkation the measures which it considers appropriate to secure the surveillance of persons who are not protected by vaccination, and who arrive on a ship that had no smallpox on board, but left a local area where smallpox was epidemic less than 14 days before Vaccination of such persons may be performed

ARTICLE 43

It is recommended that ships calling in countries where smallpox is epidemic, shall take all precautions possible to secure the vaccination or revaccination of the crew and passengers

It is also recommended that Governments should make vaccination and revaccination as general as possible, especially in ports and frontier regions

Airport quarantine restrictions.

In order to check the possible spread of smallpox by aircraft, legislation has been laid down by the International Sanitary Convention for Aerial Navigation (1933, amended 1944), and Article 35, which relates to smallpox, reads as follows.

ARTICLE 35

(a) If there has not been a case of smallpox on board, no sanitary measure may be carried out save in the case of persons who have within 14 days left a local area where

smallpox is epidemic and who, in the opinion of the sanitary authority, are not sufficiently immunized. Such persons may be subjected, without prejudice to the terms of Article 52, to vaccination, or to surveillance, or to vaccination followed by surveillance, the period of which shall not exceed 14 days from the date of arrival of the aircraft.

(b) The following measures are applicable if there is a case of smallpox on board

1. medical inspection,
2. the sick shall be immediately disembarked and isolated,
3. *other persons reasonably suspected to have been* exposed to infection and who, in the opinion of the sanitary authority, are not sufficiently protected by recent vaccination, or by a previous attack of smallpox, may be subjected to vaccination or to observation or to surveillance, or to vaccination followed by observation or surveillance, the period of observation or surveillance being specified according to the circumstances, but in any event not exceeding 14 days, reckoned from the date of arrival of the aircraft,
4. linen, personal effects, and other articles which the sanitary authority considers to have been recently infected, shall be disinfected,
5. the parts of the aircraft which have been occupied by persons suffering from smallpox and which the sanitary authority considers to be infected shall be disinfected

For the purpose of this Article "recent vaccination" shall be taken as meaning *evidence of successful vaccination not more than* 3 years or *less than* 14 days previously, or evidence of an immune reaction

Recommendations of the British Ministry of Health.*

In an official publication entitled a *Memorandum on Smallpox* (1938) the Ministry of Health issued the following instructions summarizing the procedures which should immediately be adopted by a Medical Officer of Health in the event of smallpox appearing in his district:

1 The Medical Officer of Health should visit the patient with the medical practitioner in charge of the case with a view to satisfying himself as to the diagnosis If the patient is found to be suffering from smallpox, he should at once be removed to a smallpox *hospital*

2. The Medical Officer of Health should report the matter forthwith, preferably by telegram, to the Ministry of Health, as required in London by Article 14 (5) of the Sanitary Officers Order, 1926, and outside London by Article 17 (7) of the Sanitary Officers (outside London) Regulations, 1935

3 Vaccination or revaccination should be offered to contacts In doing so the following considerations should be kept in mind The duration of the incubation of smallpox is about 12 days counting to the onset of illness, that is, an average period of 14 days before the outcrop of the characteristic focal rash If this period is divided into three intervals comprising 7 days, 3 days, and 4 days, then past experience suggests that a *successful vaccination* in the first interval will wholly prevent the attack, in the second it will more or less modify the eruption, and in the last it will have no influence on the development and course of the disease The pertinent date is not when vaccination is performed but when the reaction begins, if the reaction, which should be manifest on the third or fourth day, is delayed by any cause, then the rise of immunity is deferred For this reason prevention can only be ensured by vaccination done within a day or two of exposure and followed by a normal reaction

4 Contacts should be kept under medical surveillance for a period of 16 days after the last exposure to infection For this purpose it is seldom necessary or desirable to isolate them in their homes

5 The Medical Officer of Health should at once inform the Public Vaccinators and Vaccination Officers of the name and address of every case of smallpox as it occurs, in order that all practical methods may be taken to secure prompt vaccination or revaccination of persons willing to avail themselves of this protection

County Councils, County Borough Councils and Metropolitan Borough Councils are responsible for the administration of the Vaccination Acts in their respective areas, and in a county district the Medical Officer of Health should report his action to the County Medical Officer of Health

* Current American practice does not appear to differ in any important respect (see A P H A , *Control of Communicable Diseases*, 1945)

The Medical Officer of Health is himself empowered by the Public Health (Smallpox Prevention) Regulations, 1917, to vaccinate or revaccinate contacts

6 The infected house and its contents, together with the clothing of all persons known to have been in close contact with the patient, should be disinfected In variola minor a less stringent standard of disinfection may be adopted at the discretion of the Medical Officer of Health.

7. It is important that all doubtful cases of the disease should at once be seen by the Medical Officer of Health, and, with this end in view, medical practitioners should be notified of the presence of the disease in the district, and invited to inform the Medical Officer of Health promptly of any cases suspected to be smallpox

8 In this connection the possible confusion with chickenpox should be borne in mind, as should also the fact that this disease can be made compulsorily notifiable by the Local Authority on the advice of the Medical Officer of Health, in accordance with the procedure set out in Section 147 of the Public Health Act, 1936, and Section 305 of the Public Health (London) Act, 1936

9 The Medical Officer of Health should notify the Medical Officers of Health of adjoining areas of the occurrence of smallpox, in order that they may take any precautionary measure which the circumstances require

10 In order to ascertain the source from which the case of smallpox derived infection, careful inquiry should be made concerning the movements of the patient during the three weeks preceding the commencement of his attack, and particularly on the 12th, 13th and 14th days prior to the onset of his illness. In this way, a case of smallpox which has not previously been recognized may be discovered

11 Public Vaccinators and Medical Officers of Health can secure a gratuitous supply of lymph on application by letter, telegram or telephone[1]

[1] Lymph is available from the major public health laboratories throughout the country.

CHAPTER XXVIII

THE VARIOLA-VACCINIA VIRUS: ANIMAL EXPERIMENTS

A. ANIMAL EXPERIMENTS WITH VARIOLA VIRUS

In this section we shall describe animal experiments carried out with the virus that can be isolated from cases of true smallpox (variola major). This we shall refer to simply as "variola virus."

Monkeys

A number of persons has succeeded in transmitting the causal agent of variola major to monkeys, usually employing the contents of vesicles or pustules of cases as inocula. The infection can be passaged, the original variola becoming transformed into vaccinia (e.g Zuelzer, 1874,[1] Copeman, 1893–4 *a*, *b*, 1902–3; de Haan, 1896, Park, 1902; Roger and Weil, 1902, Magrath and Brinckerhoff, 1904 *b*; Brinckerhoff and Tyzzer, 1906, Gauducheau, 1911; Blaxall, 1923, Gordon, 1925, Clearkin, 1928–9, Yaoi, 1936 *b*, Horgan, 1938). *M. rhesus*, *M. fuscatus*, cynomolgus, and cercopithecus monkeys have been employed, and the orang-utan is susceptible (Brinckerhoff and Tyzzer, 1906).

Routes of inoculation.

Monkeys can be infected by all the common routes. After application of virus-containing material to the scarified skin, or intradermal injection, typical lesions, which will shortly be described, develop. After intravenous injection, a generalized eruption may follow in about 5 days After corneal inoculation, a typical keratitis, closely resembling that of vaccinial infection, develops. Virus may be applied to the scarified lip, nose, or palate, when pustules develop (Brinckerhoff and Tyzzer, 1906). The same authors found that infection followed inhalation of virus, or intratracheal injection, pneumonia and generalized lesions might develop

Course of the disease after inoculation of the skin The animal is usually febrile, the temperature being maximal at the end of the first week, but returning to normal by the end of the second. The primary skin lesion can usually first be noted from the 3rd to the 5th day after inoculation as a slightly red indurated crusted area By the 7th to 8th day it is much larger, measuring up to 3 cm in diameter, and there is a central crust The lesion gradually diminishes, so that the crusts have desquamated by the 12th to 14th day The lymph glands are usually enlarged on the 4th to 5th day

Comparison of variolar and vaccinial lesions after dermal inoculation Copeman (1893–4 *a*, *b*) reported that the following differences can be observed in rhesus monkeys inoculated with variola and vaccinia viruses

(*a*) The variolar lesions are more or less crusted *de novo*

(*b*) There is less vesiculation with variola than vaccinia.

(*c*) With variola a generalized eruption may appear after 9 to 11 days, and cover the whole body.

(*d*) The final scab is not so raised in variola

(*e*) T[illegible] ained, in variola

(*f*) [illegible] less active, have suf- [illegible] not fatal

Horgan and Haseeb (1939) report that the potency of variolous material for the skin is only 1/1,000, that of vaccinia is considerably more

[1] References are appended at the conclusion of Ch XXXV, p. 403 et seq

Generalized lesions in variola infection. A generalized eruption, after infection by various routes, sometimes develops about the 8th to 11th day, and lasts for about 5 days (see in particular Brinckerhoff and Tyzzer, 1906). The face is most commonly involved, and later the wrists, scrotum, anus, palms, soles, inner aspects of the arms and thighs are affected. Such generalization is unusual (Blaxall, 1930).

Pathology of the skin lesions.

A. Primary. Brinckerhoff and Tyzzer (1906) give the following description of the histological changes in variolar infection of the monkey's skin In the earlier lesions (first 3 days) degenerative changes begin in the lines of scarification, Guarnieri bodies can be found in the malpighian cells. From the 3rd day onward there is extensive degeneration of epithelial cells, this is preceded by swelling and proliferation At the same time fluid is found between the epithelial cells, producing cavities. A dissolution of the swollen and degenerate epithelial cells aids in the formation of vesicles. Polymorphs increase in number and convert the vesicle into a frank pustule. The lesion is now covered by a thick crust.

The corium shows proliferation and swelling of the endothelial cells of the lymphatics and blood vessels At a later stage necrosis occurs beneath the center of the lesion, and associated therewith is an increase in the endothelial cells, Guarnieri bodies can be found in these cells.

The final stage is one of repair, epithelium grows in from the sides, and up from the hair sheaths to displace the crust.

B. Generalized. Brinckerhoff and Tyzzer (1906) report that the epithelial changes are similar to those in the primary eruption. The reaction in the corium is, however, very much less pronounced, there being no edema or necrosis, but only enlargement and proliferation of the endothelial cells, there is infiltration with polymorphs.

Rabbits

Variola cannot usually be transmitted to the rabbit by the dermal or intradermal routes directly from the human patient, although Risel (1919), Clearkin (1928–9), and Amies (1932) have reported positive results; usually a number of transfers are needed before the virus "takes" on the rabbit's skin Recently, Horgan (1938) obtained pustules after inoculation with human material, but passage by the dermal or intradermal route did not succeed. Rabbits can be directly infected with variola by other routes, however, e g, the anterior chamber (Komiyama, 1932), the testis (see Bachmann and Biglieri, 1922, Horgan, 1938), and, of course, via the cornea (Paul's test, see Ch. XXVII).

Calves

It is generally conceded that variola virus cannot usually be propagated in the skin of calves directly from the human patient, *repeated transfers through the calf* may, however, succeed After passage through monkeys or rabbits, the virus produces a slight lesion on dermal inoculation of the calf, if dermal passage is then continued, lesions develop

Other Animals

Teissier *et al* (1931) failed to produce any definite lesions in asses by dermal or intratesticular injection, earlier, however, Chaumier and Belin (1913) had claimed *that variola could be passed directly.*

Pigs may develop a slight local lesion after variolization of the skin, but the infection cannot be passed in series (Wurtz *et al*, 1913) More recently, Teissier *et al.* (1931) failed altogether to transmit variola

Warlomont (1883) claimed to have infected horses by intravenous injection. No lesions were produced in young bulls by intratesticular or dermal inoculation

CHAPTER XXVIII

THE VARIOLA-VACCINIA VIRUS: ANIMAL EXPERIMENTS

A. ANIMAL EXPERIMENTS WITH VARIOLA VIRUS

In this section we shall describe animal experiments carried out with the virus that can be isolated from cases of true smallpox (variola major). This we shall refer to simply as "variola virus."

Monkeys

A number of persons has succeeded in transmitting the causal agent of variola major to monkeys, usually employing the contents of vesicles or pustules of cases as inocula. The infection can be passaged, the original variola becoming transformed into vaccinia (e.g. Zuelzer, 1874,[1] Copeman, 1893–4 *a*, *b*, 1902–3; de Haan, 1896, Park, 1901, Roger and Weil, 1902, Magrath and Brinckerhoff, 1904 *b*, Brinckerhoff and Tyzzer, 1906; Gauducheau, 1911; Blaxall, 1923, Gordon, 1925; Clearkin, 1928–9, Yaoi, 1936 *b*, Horgan, 1938). *M. rhesus*, *M. fuscatus*, cynomolgus, and cercopithecus monkeys have been employed, and the orang-utan is susceptible (Brinckerhoff and Tyzzer, 1906).

Routes of inoculation.

Monkeys can be infected by all the common routes. After application of virus-containing material to the scarified skin, or intradermal injection, typical lesions, which will shortly be described, develop After intravenous injection, a generalized eruption may follow in about 5 days. After corneal inoculation, a typical keratitis, closely resembling that of vaccinial infection, develops. Virus may be applied to the scarified lip, nose, or palate, when pustules develop (Brinckerhoff and Tyzzer, 1906) The same authors found that infection followed inhalation of virus, or intratracheal injection, pneumonia and generalized lesions might develop.

Course of the disease after inoculation of the skin The animal is usually febrile, the temperature being maximal at the end of the first week, but returning to normal by the end of the second. The primary skin lesion can usually first be noted from the 3rd to the 5th day after inoculation as a slightly red indurated crusted area. By the 7th to 8th day it is much larger, measuring up to 3 cm in diameter, and there is a central crust The lesion gradually diminishes, so that the crusts have desquamated by the 12th to 14th day The lymph glands are usually enlarged on the 4th to 5th day

Comparison of variolar and vaccinal lesions after dermal inoculation Copeman (1893–4 *a*, *b*) reported that the following differences can be observed in rhesus monkeys inoculated with variola and vaccinia viruses.

(*a*) The variolar lesions are more or less crusted *de novo*.
(*b*) There is less vesiculation with variola than vaccinia
(*c*) With variola a generalized eruption may appear after 9 to 11 days, and cover the whole body
(*d*) The final scab is not so raised in variola.
(*e*) The temperature rise is more marked, and longer sustained, in variola.
(*f*) The variola-infected animals suffer from diarrhea, are less active, have suffused eyes, and emit an unusual odor, the disease is not fatal.

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Dermal strains of virus are usually maintained by passage through calves, sheep, or rabbits, the animals being inoculated by scarification. When the lesions reach sufficient size, the area bearing them is scraped and vaccinial pulp obtained. Other terms used to refer to this type of virus are "dermovaccinia," "dermovaccine," "dermal vaccine," "lymph," "dermal lymph," and sometimes merely "pulp" The terms "lapine," "dermal lapine," and "dermolapine" refer to dermal vaccinia from the rabbit. Sometimes passage is carried out through human beings, when the term "humanized lymph" is used

Ledingham and McClean's (1928) Strain

These authors propagated testicular and dermal strains of vaccinia by intradermal inoculation, in rabbits Virus, when passed by this route, lost most of its potency as tested by scarification Potency increased markedly, however, when tested by intradermal inoculation; for example, the dermal inoculum might develop at least 100,000 times its original virus content The histological reactions produced by this strain are described on p 316.

Armstrong's Heat-resistant Strain

Armstrong (1929 *a*) injected dermal virus intratesticularly, after each inoculation virus was heated at 37 5° C. By continued selection and propagation, he secured a strain of virus which showed an increase of several hundred per cent in the length of time it would withstand heat This virus was used in experiments on intratracheal injection, when it was found to produce a pure virus pneumonia (Armstrong and Lillie, 1929).

Noguchi's Strain of Testicular Virus

Noguchi's (1915, 1918) experiments were initiated with the object of securing a bacteria-free suspension of vaccinia. He began by injecting rabbits intratesticularly with dermal vaccine At first, passage proved difficult, but after 10 transfers the virus was successfully established The usefulness of Noguchi's method was soon recognized, and testicular virus has been widely used in all types of experiment with vaccinia virus since that date The following other terms are applied to testicular virus· "testilapine," "orchilapine," the term "neurovirus" is also used, for certain strains of virus are often passed both intracerebrally and intratesticularly. Noguchi (1918) found that prolonged testicular passage did not lower the activity of the virus for the skin, though increasing activity for the testis, he also showed that testicular virus had no more tendency to localize in the internal organs on intravenous inoculation than had dermal vaccine. Testicular passage is frequently employed to increase the virulence of dermal or other strains of virus that have become of reduced potency

Neurovaccinia

A strain of virus widely used in experimental work is one which is maintained by cerebral passage in rabbits, with perhaps occasional testicular passage To a strain of this type, the terms "neurovaccine," "neurovaccinia," or neurovirus" are applied The worker primarily associated with the study of neurovaccine is Levaditi, who with Nicolau and other co-workers has carried out much experimental work (Levaditi, Harvier, and Nicolau, 1921, Levaditi and Nicolau, 1921, 1922 *a*, *b*, 1923 *a*, *d*, *e*, Levaditi, 1924, Levaditi *et al*, 1927, 1937, 1938 *a*, *b*)

Production of neurovaccinia.

Starting with a strain of dermal virus, the French authors carried out cerebral passage in rabbits, difficulty was experienced at first, and some testicular transfers had to be interpolated, but eventually virus could be passed by the cerebral route alone Certain workers have found, however, that some strains of virus can be

(Teissier *et al.*, 1931). Teissier *et al* (1930) found that the cat was only very slightly susceptible to variola. It has been claimed that variola can be passed in buffaloes (Gauducheau, 1911). Nelson (1940 *a*) found transient lung changes in mice on nasal instillation

B. ANIMAL EXPERIMENTS WITH ALASTRIM VIRUS

Monkeys

Numerous investigators have carried out *experiments on the transference of* alastrim virus to monkeys (Green, 1915–17, Leake and Force, 1921; Blaxall, 1923, van Hoof, see Jorge, 1924, Gordon, 1925, Ledingham, 1926 *a*, Turkhud and Pandit, 1926–7, Amies, 1932; Torres and Teixeria, 1933 *a*, *b*, 1934 *a*, da Cunha and Teixeria, 1934, Torres, 1935–6) As a general rule, it has been found that lesions develop after application of virus directly to the scarified skin, and after intradermal injection, the reaction is similar to that produced by variola virus (*vide supra*), histologically, however, the inclusion bodies may be differentiated (see p. 340) Monkeys can also be infected by intravenous injection, when skin lesions develop on the face and limbs

It would appear that on occasions some difficulty may be realized in transmitting the infection (Ledingham, 1926 *a*, Turkud and Pandit, 1926–7, Horgan and Haseeb, 1939)

Rabbits

A number of authors has tested the susceptibility of rabbits to alastrim virus (MacCallum and Moody, 1921, Moody, 1922, Blaxall, 1923, Jorge, 1924, Gordon, 1925, Ledingham, 1926 *a*, Turkhud and Pandit, 1926–7, McKinnon and Defries, [illegible] 928, Torres, 1935–6) The majority found that [illegible] skin produces either no result, or at most a [illegible], however, *definite lesions develop, resembling* those of vaccinia It appears that by the intradermal route a local eruption may develop on direct inoculation of human material (see Ledingham, 1926 *a*, McKinnon and Defries, 1928).

The rabbit's cornea is, of course, susceptible, and a positive Paul's test can often be elicited (Torres, 1935–6, Paul's test, see above)

Other Animals

Usually, lesions are not produced on application of human alastrimic material to the skin of calves (MacCallum and Moody, 1921, Blaxall, 1923, Turkhud and Pandit, 1926–7) Occasionally, however, a mild reaction may be noted (see Green, 1915–17) Cleland and Ferguson (1915) were able to secure a "take" after a few transfers Blaxall (1923) found that, using virus passed through monkeys and rabbits, definite lesions developed after a few transfers through the calf

Ledingham (1926 *a*) produced a reaction by intradermal inoculation of the guinea-pig's pad, De Korté (1904) and Green (1915–17) did not succeed in infecting these animals, however Van Breuseghem (1940) adapted virus to mice after rabbit passage

C. VACCINIA VIRUS

Variants of Vaccinia

Vaccinia exists usually as a virus with dermal properties, proliferating abundantly in skin, but only poorly in the brain A number of variants has been produced by passage under abnormal circumstances Findlay (1936) regards these as *Dauermodificationen* Levaditi *et al* (1940 *a*) regard EB suspensions as complexes containing variants of different potential pathogenicity and tissue affinity Adaptation to a particular host favors one variant at the expense of others

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more readily converted into neurovaccinia than was the case in Levaditi's experiments (see p. 317). Blanc and Caminopetros (1923) actually claimed that one corneal passage of dermal vaccine was sufficient to convert it into neurovaccinia Levaditi and Voet (1937) have shown that egg passage rapidly conferred on 2 dermal strains the properties of neurovaccinia. A feebly encephalitogenic mixture of dermotropic EB's and a few neurovaccinal EB's became transformed into fully virulent neurovaccinia by egg passage (Levaditi *et al.*, 1939 *a*).

Some properties of neurovaccinia.

During the first 100 intracerebral passages, 85 per cent. of rabbits died after 4 to 7 days, and 77 per cent. after 4 to 6 days; 4 per cent. died on the 3rd day following injection. During the second 100 passages, 72 per cent. died on the 4th or 5th day, and 14 per cent. on the 3rd day; this indicated a considerable increase in virulence (Levaditi, 1924). It is this property of increase in activity over dermal lymph that has caused neurovaccine to be so widely used in experimental work.

Neurovaccinia can be transmitted to numerous animals, and Levaditi and Nicolau (1923 *a*) summarized these results as follows: the virus is infective for rabbits, guinea-pigs, rats, mice, calves, and monkeys by the cutaneous route, it is infective for rabbits, guinea-pigs, cats, and monkeys by the cerebral route. Smith, Horgan, and Haseeb (1941) reported on the properties of the Lagos mouse neurovaccinia. This was of high virulence for the mouse and rabbit cerebrally, but retained full dermotropic potency.

Other properties will be alluded to below.

The tissue affinities of neurovaccinia.

Although Levaditi originally held that neurovaccine was endowed predominantly with an affinity for the ectoderm, this opinion has been revised as follows (Levaditi *et al.*, 1938 *a*) In vaccinia virus there are held to be 2 components, "E" and "M", "E" affects the ectoderm, and "M" the mesoderm. Dermal virus, for example, does not readily produce a reaction on cerebral injection, nor does it produce a reaction in the pleural and peritoneal cavities on local injection. In this case, the "E" component completely masks "M." Neurovaccine, however, produces severe cerebral changes, and the pleuroperitoneal reaction. With this strain, "M" is the dominant component. During the conversion from dermovaccine to neurovaccine, the relative content of these 2 factors undergoes an alteration. Probably the receptivity of the reticulo-endothelial systems of the central nervous system of the host plays the part of a selector in securing this change. They have also shown that the encephalitogenic power of a strain may vary from *récolte* to *récolte*; further, some rabbits are relatively insusceptible to vaccinia by intracerebral injection, possibly due to a previous attack of rabbit-pox (1938 *b*)

The biological nature of neurovaccinia.

Elsewhere it has been described how rabbits may sometimes develop a spontaneous infection known as rabbit-pox (see p 323). Although the causal agent of this condition is evidently related to the variola-vaccinia group, it appears to be independent Now, since rabbits can contract such a disease spontaneously, it may be asked whether neurovaccinia is not in reality a strain of rabbit-pox virus modified by repeated passage (see Borrel, 1936)

Levaditi and Voet (1937) were able to show that 2 bovine viruses could be transformed into neurovaccinia by egg passage, without the aid of rabbits. It is unlikely, therefore, that rabbit-pox virus is the original source of neurovaccinia

Levaditi regarded neurovaccinia as a fixed virus, for passage on the rabbit's skin did not alter it back to dermovaccine, nor did passage through the human skin effect any change (Nicolau and Poincloux, 1924). This view has been recently upheld by Levaditi, as he has shown that 5 transfers by dermal inoculation of

monkeys induced no alteration in the characters of the virus (Levaditi *et al*, 1937). Douglas, Smith, and Price (1929), however, maintained that testicular passage altered the properties of neurovaccinia to a certain extent. First, intradermal reactions were much more constantly elicited Second, the testicle-passed virus had a much greater virulence for the peritoneum and bladder. Third, generalization was induced with greater constancy.

It may be said that there is some doubt in the minds of many whether neurovaccinia is as distinct from dermal virus as is, for example, fixed from street rabies virus It is felt that the greater activity of neurovaccinia may be due merely to the fact that it proliferates much more luxuriantly in the brain than does dermal virus in the skin. Levaditi (1939*a*) has shown that the number of EB's in the minimal infective dose is much less in neurovaccinia than in dermal vaccinia.

Cultured Virus

Of recent years strains of both dermo- and neuro-virus have been grown in tissue culture and in the egg (see Chs XII, XIII). Although certain changes may be induced by growth *in vitro* or in the egg, as a general rule no radical differences in properties develop.

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ROUTES OF INOCULATION

Dermal inoculation.

Rabbits are readily infected by direct application of vaccinia virus to the freshly shaved skin, and by scarification of depilated skin. The area selected is usually the back, but Tyzzer (1904) recommended the internal surface of the ear. It is convenient to rub the virus into the skin by means of a glass rod. The lesions usually first appear from the 3rd to the 7th day, the earliest evidence of reaction being red papules Vesicles, which soon coalesce, are usually noted by the 5th day; pustulation occurs and scabs form. After cutaneous inoculation, the higher the dilution of virus the larger is the vesicle produced, and the fewer vesicles there are, the number of vesicles in proportion to the amount of virus increases in the higher dilutions (Groth and Munsterer, 1935)

Titration of virus. Virus is often titrated by application to the scarified skin, and the following instructions of Gordon (1925) should be followed

(*a*) Rabbits should weigh 1,500 to 2,000 gm
(*b*) The test fluid is diluted in saline, e g, from 1/1,000 to 1/1,000,000.
(*c*) A drop of the test fluid is placed on the shaved skin, and scratches made through the drop with a sharp surgical needle.
(*d*) The lesions become evident from the 3rd to 7th day.
(*e*) To remove pulp the animal is killed and bled, the carcass is left for a period, to allow the remaining blood to clot The shaved area of the skin

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is removed, and the lesions are scraped off. The pulp is weighed and any desired amount of saline added to give a definite known dilution Finally, it is placed in a bottle with 10 per cent. of ether and stored in the cold

Pathology.

1. *Tyzzer's* (1904) *experiments* Tyzzer examined the histological findings after scarification of the internal surface of the rabbit's ear. After 4 to 8 days the skin was reddened, the surface being elevated and somewhat opaque; if pricked, lymph exuded. After a further 1 to 2 days, the lesion increased in extent, and severe itching was experienced Histologically, after 3 days the skin was seen to be increased to 2 or 3 times its normal thickness The blood vessels and lymphatics were dilated, the endothelium of the smaller vessels was proliferated. Numerous polymorphs and lymphocytes infiltrated the tissues, and there was an increase of connective tissue cells. The malpighian cells showed vesicular change, Guarnieri bodies could be found in the cells outside the vesicles The surface of the lesion was covered by a crust composed of the horny layer with polymorphs and fibrin

2. *Levaditi and Nicolau* (1923 *a*) compared the skin lesions produced by neuro- and dermo-vaccines. Neurovaccine produced an erythematous reaction which, on the 3rd day, was followed by a *more or less confluent papular eruption.* The lesions of neurovaccine were more swollen, redder, more infiltrated, more intense, and deeper. When the neurovaccinal papules were beginning to become pustular, the dermovaccinal had progressed to umbilication and suppuration Later, the neurovaccinal lesions became confluent, the surface underwent dry necrosis, and a thick scab formed which lasted for weeks, the dermovaccinal lesion, on the contrary, scabbed quickly after becoming pustular Microscopically, the dermal strain showed a greater tendency to involve connective tissue

3. *Ledingham* (1924) studied the histology of the rabbit's skin after infection by scarification, with a view to determining the primary tissue response to vaccinia (see also p 329) The earliest change was an involvement of the capillaries in the papillary region, the deep dermis being only slightly involved The malpighian cells and hair follicles were severely disorganized by subjacent exudate Diffuse and perivascular infiltrations were the predominant feature of the response

4. *Andervont* (1926 *b*) drew attention to the formation of the vaccinal vesicles, at first subdivided into compartments by elongated epithelial cells, but later becoming monolocular owing to destruction of these cells He also referred to changes in the corium, such as infiltration with polymorphs, capillary dilatation, and thickening of the capillary walls

5. *Amies's* (1938) *experiments with crude lapine* Amies described the lesions produced by crude lapine inuncted through the shaved skin Almost no lesions were found at 24 hours, but after 48 hours the epidermal cells had proliferated to form a layer 6 to 10 deep Many cells showed early "balloon" degeneration Small eosinophilic bodies less than 1 μ in diameter were found in the cytoplasm In some cases these bodies were larger, and the cells containing them showed disintegration, these necrotic areas were invaded by polymorphs that surrounded the eosinophilic bodies After 72 hours round-cell infiltration of the dermis, first seen earlier, had become more marked The malpighian layer was now 15 cells deep in some areas, much "ballooning" being present Inclusions could only be found in a few cells at the edges of the large areas of eosinophilic necrotic material At their bases these areas were invaded by polymorphs and other inflammatory cells, the hair follicles also showed invasion by polymorphs and cellular proliferation

After 4 days the epidermis was almost completely replaced by strongly acidophilic material Inclusions could be found in the hair follicles, both in the more healthy cells and lying free between those that were degenerate The round-cell infiltration of the dermis was more pronounced

6. *Amies's* (1938) *experiments with elementary bodies* Amies also studied the

reaction after application of homogeneous suspensions of elementary bodies (see p. 329) to the shaved skin.

Compared to the reaction produced by crude lapine, these lesions were seen to be milder; inclusions occurred more frequently, and dermal infiltration was much less marked.

The vaccinial inclusion first developed as a small eosinophilic granule about 0.5 μ in diameter, in the perinuclear space of the epidermal cell, 48 hours after infection. Two or more such bodies were often seen in any one cell, in the later stages these granules were considerably larger. Some cells were literally packed with bodies, and it was noted that the nuclei of cells containing inclusions remained intact till a late stage.

Intradermal injection.

Rabbits can be infected readily by intradermal injection, this route was probably popularized first by Novotny and Schick (1910). Characteristic papulopustular lesions, which eventually undergo scabbing, develop in 3 to 5 days

Vaccinia virus can be simply titrated by intradermal injection of serial dilutions from 1/10 to 1/1,000,000, an average titer of dermal virus being 1/10,000 to 1/100,000 Ledingham (1924) found that with lymph diluted 1/100, the reaction might not be visible for 3 days, although with lower dilutions it might be evident in 24 hours, the reaction after inoculation of a 1/10,000 dilution did not appear for 3 to 6 days (see also Clearkin, 1930) Titrating EB suspensions, Henderson and McClean (1939) found the titer by scarification 1 100 of that obtained by intradermal inoculation

After intracutaneous injection, it was found that no virus could be demonstrated locally from 20 minutes to 20 hours after the inoculation, but thereafter virus increased gradually to reach a maximal concentration (Widelock, 1938) He found that the same maximum was reached whether the original inoculum was diluted 1/100 or 1 1,000,000 As might be expected, the greater the concentration of virus injected the sooner was this maximum attained He showed also that the size of the reaction varied with the bulk of the inoculum. The severity of the reaction did not depend on the quantity, but on the concentration of the inoculum, for example, the injection of 0 01 c c or 0 5 c c of the same concentration produced a reaction of equal intensity

Groth and Munsterer (1935) reported that when rabbits were inoculated with falling dilutions of vaccinia, the area of infiltration did not decrease in proportion to the degree of dilution. In fact, with reference to the amount of virus, the area was considerably increased in higher dilutions

Ledingham (1926 *b*) showed that the reaction to vaccinia injected intracutaneously could be annulled, or very considerably reduced, by simultaneous injection of India ink This effect also operated if virus was injected into areas of skin previously infiltrated with ink

Passage of vaccinia by the intradermal route Ledingham and McClean (1928) carried out experiments on the propagation of testicular and dermal vaccinia by the intracutaneous route After passage the virus lost most of its potency when tested by scarification For example, material giving a titer of 1 1,000 to 1 10,000 by intradermal injection, only produced a reaction on scarification when diluted 1 10 Barg and Rudenko (1935) confirmed these observations

Pathology

1. *Ledingham* (1924) carried out an extensive series of observations on the reaction of the rabbit's skin to the intradermal inoculation of lymph.

After 6 hours polymorphs were found to infiltrate the cells of the hair follicles The venous channels of the dermis were congested and often filled with poly-

morphs. The dermis (excepting the papillary region) was infiltrated with polymorphs, and there was slight edema of the tissue spaces.

After 24 hours the polymorphs in the follicles showed karyorrhexis; in the dermis there were more polymorphs. The endothelial cells of the capillaries in the papillary region and overlying skin muscle were "frayed-out," and showed occasional mitoses. Endothelioid cells from the adventitia were engulfing dead polymorphs.

After 2 days the malpighian cells showed some infiltration with polymorphs The dermis showed the beginning of a division into 3 layers The central layer showed degenerate polymorphs and necrotic or hyaline tissue fibers; there were occasional large vessels, the walls of which were infiltrated with polymorphs. Fibrin threads could also be seen; some of the vessels in the central zone showed complete coagulation necrosis of the walls, with a surrounding fibrinous exudate Above and below this central zone were dense infiltrations of polymorphs, adventitial cells engulfing these, and small vessels from whose sheaths adventitial cells were produced.

After 3 days definite chromatolysis and swelling of some of the malpighian cells were observed In the dermis the central zone was very distinct, and consisted of finely comminuted polymorph chromatin and isolated necrotic vessels There was active proliferation of adventitial cells over the skin muscle, and through the perimysium.

After 4 days "ballooning" of the malpighian cells was seen, with some chromatolysis The deeper parts of the hair follicles were often replaced by young vascular tissue with many adventitial cells showing mitoses In the dermis the main change was that new vascular tissue was being formed actively in the boundary zones

After 7 days the malpighian cells were shrunken and showed numerous mitoses In the dermis the central zone was being invaded by the newly formed vascular tissue from above and below. In many places, adventitial cells showed perivascular cuffing, mitoses were common, and a few plasma cells were seen.

After 10 days the only abnormality in the epidermis was the destruction of the deeper portions of the hair follicles. In the dermis the central zone was much thinner, and young connective tissue fibers were being laid down at its margins. The vessels in the peripheral zones were widely cuffed.

After 15 days there was follicular regeneration, the central zone being now entirely replaced by histiocytes and young connective tissue fibers

2 *Lesions produced by Ledingham and McClean's* (1928) *strain* The dermal reaction was seen to be essentially similar to that produced by ordinary lymph, the primary attack being on the vascular endothelium, later, there was an infiltration with polymorphs and a mobilization of histiocytes. Necrotic changes took place in these cells, and the nuclear debris occupied a large area of the mid-dermis There was evidence of reticulo-endothelial involvement in the perivascular sheath, also, more diffusely, in the neighborhood of the larger vessels of the upper papillary layer and skin muscles, and also throughout the skin muscles and areas of dermis peripheral to the central lesion Hemorrhagic necroses were frequent along the course of vessels running up through the mid-dermis to the papillary layer The lymph glands draining the inoculated areas were red, swollen, and might show definite discrete hemorrhages.

3 *Thompson and Buchbinder's* (1932) *experiments* These workers carried out a series of tests using the following strains two nonencephalitogenic strains of virus (Noguchi's testicular virus and the NY strain of calf lymph), Mulford's [illegible] passage in rabbit brains, and finally Leva- [illegible] as follows The two nonencephalitogenic [illegible] age, and only slight edema and necrosis Resolution began after 144 to 168 hours Secondary lesions were few, and occurred

after about 144 hours, the animals never died as a result of these injections. The reactions produced by the 3 encephalitogenic strains were, however, very different. Hemorrhages occurred with Mulford's rabbit brain strain and Levaditi's strain; edema and necrosis were severe in all cases. Resolution began after 168 to 192 hours with both Mulford's strains, but after 144 to 192 hours with Levaditi's After 96 hours secondary lesions were marked and extensive with Mulford's strains, being only slight with Levaditi's virus. Death occurred frequently after inoculation of Mulford's rabbit brain strain, but not with the other two.

Subcutaneous injection.

Rabbits can be infected by the subcutaneous route, and if the dose is sufficiently large, a generalized eruption may develop It is said that if virus is injected in a large volume of water, a greatly swollen infiltrated mass develops giving a large yield of virus (Blanc and Caminopetros, 1924 *a*).

Ledingham (1924) studied the pathological features of injection of lymph into the groin By the 3rd day a hard mass had developed near enlarged glands. On the 6th day hemorrhage was seen in the subcutis, and there were yellowish tracts of exudate overlying the muscle tissue, and extending to the dermis above the skin muscle These tracts consisted of polymorph debris and adventitial cells containing polymorphs; they were surrounded by large areas of embryonic vascular tissue containing newly formed capillaries Some capillaries contained blood, while extensive hemorrhages were seen near others Between the capillaries there were large numbers of adventitial cells There was no involvement of the epidermis

Intracerebral injection.

There is considerable individual variation between vaccinial strains in the facility with which they can be transmitted to rabbits by intracerebral injection, and many authors have referred to this point (see Bachmann and Biglieri, 1923 *b*, Zurukzoglu, 1927, Herrmar [illegible]

For examp [illegible] of experimenters have failed to prod [illegible] and have been unable to carry on [illegible] 8–9, Grinker, 1930–1, Beattie and Potter, 1934). Pette and Kornyey (1933) could not discover changes resembling those of human postvaccinal encephalitis after injection into the cisterna magna Other workers, however, have been more successful For example, Marie (1920) carried out intracerebral injection of dermal vaccinia and passed the virus in series. He found that the virus spread to the lowest part of the cord, and the animals usually died between the 5th and 8th day. Condrea (1922 *b*) found that rabbits injected intracerebrally with dermal lymph developed nervous symptoms, and that the virus could be passed and become of exalted virulence.

Gordon (*Report*, 1928) produced encephalitis in rabbits by an intracerebral, followed by an intravenous, injection of lymph Virus could usually readily be demonstrated in the brains of rabbits dying within 10 days, but not in those dying after 12 to 13 days. McIntosh (1928) produced encephalitis by using a highly virulent dermal lymph. Blanc and Caminopetros (1931) found that corneal passage of dermal lymph resulted in the production of a strain which, on intracerebral injection, produced encephalitis.

Although the dermal strain of the New York Health Department produced no symptoms on intracerebral injection, Mulford's lymph induced a severe and [illegible]

injection Using the same strain in 1929, however, success was achieved.

A considerable advance was made in the study of vaccinia virus when it was shown that intracerebral passage could be readily carried out if the original inocu-

lum had been passed intratesticularly; further, success was more easily secured if an occasional testicular passage was interpolated in the cerebral series (Levaditi, Harvier, and Nicolau, 1921, 1922). It is now firmly established that rabbits develop meningo-encephalitis after injection of neurovirus, whether the inoculum is infected testis or brain (McIntosh and Scarff, 1930, Hurst and Fairbrother, 1930, Spooner, 1930).

Pathology.

A number of workers has described the pathological changes induced by neurovaccinia or virulent dermal virus (Condrea, 1922 *c*, Levaditi, 1924, McIntosh and Scarff, *1930, Spooner, 1930, Hurst and Fairbrother, 1930; Bardach, 1931; Force,* Beattie, and Lucia, 1939). The meninges are congested, contain small hemorrhages, and there is perivascular cuffing with mononuclear and plasma cells Guarnieri bodies occur in nerve and ependymal cells. The brain proper shows some perivascular cuffing, and degeneration of nerve cells Miliary abscesses may occur. McIntosh (1928) described the changes in his experiments as like those in human postvaccinal encephalitis, but Spooner (1930) found no demyelination. The changes are mainly those of a meningitis, and to a less extent of encephalitis

The production of encephalitis by peripheral injection.

Mainly with a view to investigating the etiology of human postvaccinal encephalitis (see Ch. XXXV), a number of workers has tried to produce encephalitis in rabbits by injecting vaccinia peripherally. Certain authors have failed to produce any evidence of encephalitis after dermal, subcutaneous, corneal, intravenous, or intratesticular injection of various strains of virus (Zurukzoglu, 1927; Demme, 1928, Pette and Kornvey, 1933). Further, the effect of various traumata and injections directed toward causing the cerebral localization of the virus has been studied with negative results by the following authors Berger (1929) showed that *injury to the skin, and anesthesia, did not cause the virus to produce encepha*litis after dermal inoculation Hurst and Fairbrother (1930) were not able to produce encephalitis in rabbits by intradermal inoculation of ordinary lymph or neurovaccine, despite injection of nervous tissue intracerebrally, or horse serum intrathecally, in an attempt to localize the virus Szymanowski and Zand (1931) tried to facilitate the passage of virus to the brain after intratesticular injection by injecting rabbits with trypan blue to blockade the reticulo-endothelial system, no success was achieved.

Other workers have, however, been successful in their attempts to cause localization of virus in the brain by peripheral injection For example, although normally the intravenous injection of neurovaccine does not cause virus to appear in the brain, the intracerebral injection of saline or broth secures localization (Levaditi and Nicolau, 1923 *a*) Evidence of cerebral involvement after peripheral inoculation has been furnished by Blanc and Caminopetros (*1924 b, 1926*). After corneal, *cutaneous, subcutaneous, or intravenous inoculation of lymph,* animals developed encephalitic symptoms There was usually a rise of temperature, and if animals were killed at this time virus could be recovered from the brain It has been reported that encephalitis may sometimes develop in rabbits inoculated corneally with all types of virus (Levaditi and Nicolau, 1926) McIntosh and Scarff (1930), using a virulent neurovaccine, sometimes noted *meningo-encephalitis after intra*dermal inoculation, after intravenous injection this occurrence was somewhat more common. Grinker (1930–1) reported that after intratesticular injection a fatal meningo-encephalitis might develop

Corneal scarification.

After application of virus to the scarified cornea of the rabbit, an acute keratitis develops in 2 to 4 days The surface of the cornea becomes quite "steamy"

and, later, opaque. There is usually a considerable degree of mucopurulent conjunctival discharge The cornea shows a decreased transmission of light *in vitro* (Little *et al.*, 1947).

Pathology.

One of the best descriptions of the pathological features of vaccinial keratitis is that of Tyzzer (1904). Within 36 hours the scarification wound marks are obliterated by growth of epithelium. On the second day after infection the corneal surface is seen to be uneven and elevated at the scarification marks From this time onward some slight circumcorneal congestion is apparent. The cornea becomes "steamy," and there is purulent conjunctival exudate Histologically, after 4 to 8 hours the appearances do not differ from those due only to injury. After 16 hours inclusions occur in the vicinity of the nucleus of the epithelial cells (see also, Levaditi and Schoen, 1935) Some polymorphs can be found infiltrating the substantia propria After 50 hours the epithelium near the scarification marks is much proliferated and many inclusions can be found, in other areas the epithelium is very thin Some epithelial cells are seen to be included by others. Polymorphs are now quite numerous After 3 days there is still further epithelial proliferation, and Guarnieri bodies occur at a greater distance from the scarification Eosinophilic inclusions can be found in the fibroblasts of the substantia propria (Rhodes and van Rooyen, 1937 *a*). After 5 to 6 days the epithelium is 5 to 6 times its normal thickness, Guarnieri and fibroblastic bodies are less frequent, and many epithelial cells are included. At this stage, many of the infiltrating polymorphs become degenerate and disintegrate, liberating their granules From the 9th to 11th day Guarnieri bodies may be found in the epithelium, but are absent in some specimens

Intratesticular injection.

The rabbit can be infected with vaccinia by intratesticular injection, a method first introduced by Noguchi (1915, 1918), the value of which is now firmly established As a general rule, dermal lymph produces only a slight reaction; continued passage results, however, in the production of a strain with high affinity for testicular tissue

Noguchi (1915) found that after 48 hours the virus had increased approximately a hundredfold over its titer at the end of the first day and continued to increase for a few days

The value of the method was confirmed by Condrea (1922 *a*, *b*) and Ohtawara (1922). The peritoneal cavity may contain clear, straw-colored exudate of semigelatinous consistency (see Douglas, Smith, and Price, 1929).

Pathology

After intratesticular injection Noguchi (1915) found the following changes During the first 24 hours few changes were found in the testis, although there were microscopic foci of infiltration with polymorphs, and exudate in the interstitium After 48 hours the testis became swollen, congested, and edematous There was a heavy leukocytic infiltration in the interstitium and tubules, the tubules were filled with hydropic cells, after 3 days, the infiltration was even more marked Naked-eye, after 4 days, the testis was purplish-red, and spotted here and there with irregular yellowish areas, the organ was friable and there was exudate in the tunica vaginalis Histologically, at this period, there were several groups of tubules which had undergone total necrosis After 6 days the reaction had decreased somewhat, for there was less edema and cellular infiltration, by the 7th day the infiltration was resolving more rapidly By the 10th day the organ was reduced in size below its normal dimensions From the 12th to 18th days the organ became even more shrunken, and microscopically there was loss of testicular cells and collapse of tubules After 5 weeks there was complete parenchymal atrophy.

Intravenous injection.

This route of injection is most commonly used in order to produce generalized vaccinia. This syndrome is fully discussed on p. 321 and no more need be said here, except to quote some recent interesting work of Gordon (1939*a*), who recorded experiments on the production of fibrositis and arthritis with vaccinia virus Rabbits were injected intravenously on two occasions, at a 4-day interval If the dose of virus was too large, a polyarthritis developed, usually, however, there was a mono-arthritis of one hind leg. The lesion was essentially periarticular, and usually situated near the insertion of the Achilles tendon. If the rabbit was killed in the early stages, the subcutaneous tissues were filled with a serofibrinous exudate, at first, the virus was present in a dilution of 1:1,000,000, later only in 1.1,000, and by the 11th day it was absent. Virus was found in the popliteal gland draining the affected area, but was present in the blood and spleen only in small quantity. Histologically the lesions resembled those of rheumatic fibrositis in man.

Intraperitoneal injection.

Rabbits may be infected by the intraperitoneal route with neurovaccinia, producing an exudate in the peritoneal cavity, adhesion of the mesentery, and the formation of Guarnieri bodies in the mesentery and skin of the abdominal wall (Borrel, 1936). Douglas, Smith, and Price (1929) found that the surfaces of the gut and the peritoneum were stippled with hemorrhages.

Intrasplenic injection.

Ledingham (1924) adopted this route of inoculation in his work on the primary tissue reaction in vaccinia. At postmortem the capsule was thickened and dead white near the point of inoculation, nodules were found on the adjacent abdominal wall and the peritoneal coat of the large intestine. Histologically, the white plaques consisted of a loosely arranged reticulum of trabecular cells which extended in from the capsule and ramified through the organ At the margin there were isolated hemorrhages. The white plaque seemed to be the outermost *defensive line* of numerous primary karyorrhectic foci. These foci were immediately surrounded by actively dividing primary stem cells, and in the outer rows were many plasma cells.

Intra-oral inoculation.

Levaditi and Nicolau (1923*a*) infected the rabbit by swabbing the mouth with neurovaccinia, pustules were produced, and the saliva became infected.

Nasal instillation.

Gordon (1925) found that rabbits could be infected by this route, an acute catarrh developing after 6 to 8 days, the mucopurulent discharge contained the virus. The catarrh might spread to the conjunctival sacs. Following nasal instillation, it has been said that virus can be found in the cervical lymph after 12 hours, thereafter for 7 days virus enters the blood by the cervical lymph ducts, passing through the lymph glands *en route* (Yoffey and Sullivan, 1939)

Intratracheal injection.

After injection of neurovaccine, it was found that a rash might develop on shaved areas of skin (Levaditi and Nicolau, 1923*a*).

Armstrong and Lillie (1929) carried out experiments on the intratracheal injection of Armstrong's heat-selected strain The animals developed severe respiratory symptoms which usually proved fatal The appearances were those of virus pneumonia.

Injection by other routes.

Intrapleural injection. Bardach (1932 *b*) showed that rabbits injected intrapleurally with neurolapine died, with exudate on both the inoculated and uninoculated sides.

Intrapulmonary injection. Muckenfuss *et al* (1932) studied the effects of injecting vaccinia directly into the lungs At first the alveoli contained coagulated al-

Sciatic nerve injection Levaditi and Nicolau (1923 *a*) infected rabbits by injection into this nerve.

Injection into fetal rabbits Woolpert (1936, Woolpert and Gallagher, 1937) introduced the technique of injecting the head of the 20- to 40-day-old rabbit fetus, the uterus was delivered by laparotomy and the injection performed through the uterine wall After injection of neurovaccine, the virus disseminated widely and killed the fetus in 7 days, depending on the dose (see also Gallacher and Woolpert, 1940).

Generalized Vaccinia in Rabbits

Under certain circumstances, rabbits injected with vaccinia virus may develop lesions in many parts of the body, the condition being known as "generalized vaccinia" Numbers of authors have studied this phenomenon (Camus, 1917–18, Noguchi, 1918; Levaditi, Harvier, and Nicolau, 1921, Ledingham, 1924, 1928–9,

Hu, and Pearce, 1936 *c*). These lesions are particularly liable to develop after intravenous injection, but also after inoculation by other routes The lesions have been produced most commonly with neurovaccinia Ordinary dermal lymph only exceptionally gives rise to generalized lesions. Duran-Reynals (1929) found that about 25 per cent. of rabbits suffering from enhanced lesions due to the effect of testis extracts died from generalized vaccinia. Hasskó (1932) found that generalization was particularly apt to develop in animals subjected to repeated injections of iron acetate.

Lesions occur most commonly on the skin, often showing a tendency to localize on areas traumatized by shaving or irritants. This localization effect is often known after the names of Calmette and Guérin (1901), who injected virus intravenously and noted the development of lesions on the shaved skin Similarly, after intravenous injection, keratitis may develop if the cornea is scarified or exposed to x-rays (Levaditi and Nicolau, 1923 *a*)

Papules are commonly found at the mucocutaneous junctions, they also occur in the nostrils and air passages, mouth, lips and tongue, esophagus, anal mucosa, and genital region Keratitis, orchitis, and adenopathy may all occur. Lesions are often found in the thymus, spleen, and liver, and they may occur in the brain.

The pathology of generalized vaccinia

Douglas, Smith, and Price (1929) gave the following description of the naked-eye changes in generalized vaccinia The skin pock began as a small raised papule which became hard and nodular. Slightly later it became vesicular, and then umbilicated, centrally the pock was dark and punched out, due to necrotic changes

The liver and spleen showed, *in the early stages, round dull white spots, later,* there was a semitranslucent grayish zone, with a depressed white center, rarely, a narrow purple zone of congestion might form around the lesion. The lung lesions were at first punctate hemorrhagic spots, but when fully developed appeared as glistening grayish nodules, hemorrhage being seen in the center. McIntosh and Scarff (1929) regarded the changes as those of an infective granuloma affecting primarily the smaller blood vessels, there being endothelial proliferation, tissue necrosis, and later, fibrosis. (For other papers on the pathology of generalized vaccinia see Ledingham, 1924; Ledingham and Barratt, 1929, Stewart and Duran-Reynals, 1929)

Blood Changes in Infected Rabbits

It appears that workers have seldom reported on this question. Hinc (1926) described the following changes in the blood of rabbits inoculated cutaneously with vaccinia· hemolysis, and the presence of bilirubin; an invasion of normoblasts, and a lowering of the red cell count and hemoglobin level, mainly from the 3rd to 8th day; a polynucleosis began about the 4th day, and lymphocytosis about the 6th to 7th day, both these *reactions being over by the 11th day* Douglas, Smith, and Price (1929) described the following changes in 2 animals inoculated intravenously and developing generalized vaccinia: On the 2nd day after injection the number of red cells began to fall, and this continued till the 7th day, when the count was about half the normal. Then the count increased, to become normal from the 15th to 16th day. The color index was raised during the fall Normoblasts were found, especially shortly after injection. The total white count was reduced for 2 to 3 days, it then increased, to reach a maximum about the 9th day. The large mononuclears showed the greatest increase, polymorphs were increased to a lesser extent, while lymphocytes were definitely decreased, myelocytes were found from the start.

Distribution of Virus in Infected Rabbits

Brain and cord As would be expected, virus is found in the brain, and sometimes the cord, after direct intracerebral injection. It probably disappears from the brain fairly rapidly (10 days—Gordon, *Report*, 1928, see also Herzberg, 1931 *a*), on occasions, virus appears to remain for a long period—Perdrau (*Report*, 1928), in fact, finding it 7 months afterwards. Numerous authors have isolated virus from the brain, and less commonly the cord, of rabbits infected with vaccinia by routes other than intracerebral (Levaditi, Harvier, and Nicolau, 1921, Plotz, 1922, Blanc and Caminopetros, 1923, 1924 *b*, Levaditi and Nicolau, 1923 *a*, Barikine *et al*, 1924. Huon and Placidi, 1924, Lucksch, 1927, Zurukzoglu, 1927, McIntosh and Blaxall, see *Report*, 1928, Berger, 1929, Douglas, Smith, and Price, 1929, Cattaneo, 1930) This localization of vaccinia has been noted after injection of both dermo- and neuro-viruses by the following routes dermal, subcutaneous, corneal, intratesticular, intravenous, and intrasciatic

It has been found that the intracerebral injection of human brain tissue serves to localize vaccinia in the brain of infected animals (Levaditi and Nicolau, 1923 *c*, Levaditi, Lépine, and Troisier, 1928)

Cerebrospinal fluid Gildemeister and Hilgers (1930 *a*) isolated virus from the cerebrospinal fluid in 5 out of 34 rabbits after skin inoculation

Blood Vaccinia has been found in the blood of infected rabbits, although not very constantly (Ohtawara, 1922, Gildemeister and Heuer, 1927 *a*, 1928, McIntosh and Blaxall, see *Report*, 1928) It appears that an explanation for the failure of various workers to find the blood infective is now available, because Smith (1929) showed that virus present in blood, was rapidly removed by the blood cells In this way, whole blood appeared to be virus-free, but after fractionation virus was detected in the white cells He found vaccinia in the blood up to 8 days after in-

jection In an immune animal he found that it disappeared in 4 to 6 days. He concluded that this masking of virus in whole blood was due to the presence of im-
vaccinia virus was
noted that Douglas
"take up" vaccinia
virus *in vitro*. After direct intravenous injection virus could be demonstrated irregularly in the blood for up to 6 days (Douglas, Smith, and Price, 1929)

Certain experiments have also been conducted on the effect which blockading the reticulo-endothelial system with India ink or iron acetate has on the distribution of vaccinia virus in the blood (Hoen, Tschertkow, and Zipp, 1926, Gildemeister and Heuer, 1927 *a*; Goldman, 1927–8, Hasskó, 1932). It may be said that the results are at variance, and there is dubiety whether this operation has any effect in altering the occurrence of vaccinia in the blood, as compared with controls

Lymph and lymph glands After nasal instillation, virus can be found in the cervical lymph Centrifugation of this lymph at only 2,000 r p m has the effect of depositing the virus, in association with the lymphocytes, it can also be shown that lymphocytes fix virus *in vitro* (Yoffey and Sullivan, 1939) Virus may be found in lymph glands, especially in the regional glands after intracutaneous injection (Orskov and Andersen, 1938 *b*)

Other organs and fluids Vaccinia has, on occasions, been isolated from the following fluids and organs. liver (McIntosh and Blaxall, see *Report*, 1928), lungs (Ohtawara, 1922, Levaditi and Nicolau, 1923 *f*, Winkler, 1927), urine (Gildemeister and Hilgers, 1930 *a*); saliva (Levaditi and Nicolau, 1923 *a*); testis (Levaditi and Nicolau, 1923 *f*), ovary (Levaditi and Nicolau, 1923 *f*), amniotic fluid of pregnant animals (Blaker, 1936), mammary glands (Levaditi and Nicolau, 1923 *a*), adrenals, tongue, spleen, marrow, kidney, and heart (Douglas, Smith, and Price, 1929); nasal mucosa (Gins *et al*, 1929), nasal and buccal mucosae, larynx, trachea, esophagus, ileum, and bladder (Gordon, see *Report*, 1928) Pearce (1940) found virus might persist in the testis for 17 weeks after inoculation by various routes.

SPONTANEOUS VACCINIA AND RABBIT-POX

There have been a number of instances recorded where animals have contracted infection with a strain of vaccinia (or closely related virus) without, apparently, having been directly inoculated therewith In certain of these cases the virus isolated has proved to be an ordinary strain of neurovaccinia, in such instances it is probably correct to regard the condition as a neurovaccinial infection acquired spontaneously—spontaneous vaccinia In other cases the virus has been endued with unusual properties, and although resembling neurovaccinia has differed therefrom in certain respects, notably in its greater potency. On these occasions the disease is best regarded as "rabbit-pox"—a spontaneously acquired infection due to the virus of rabbit-pox

(*a*) Levaditi and Sanchis-Bayarri (1927), for instance, found that a rabbit inoculated with suspectedly syphilitic material developed a spontaneous vaccinial infection, a strain of neurovaccine being isolated The injected animal had been in the neighborhood of infected animals although it had itself never been inoculated with vaccinia

(*b*) Nicolau and Kopciowska (1929 *a*, *b*) described a spontaneous vaccinial malady in rabbits The animals suffered from a nasal discharge, conjunctivitis and orchitis were also noted, death usually occurred after 5 to 11 days, neurovaccinia virus was isolated from the brain, testis, and skin papules

(*c*) In a later communication (1931) they alluded to the presence of virus in the ovary, adrenal, lung, spleen, liver, brain, and blood In certain cases numerous vaccinial pustules were found on the lung surfaces, usually associated with a purulent pleuropneumonia Orchitis was also found In some cases pustules were present

The liver and spleen showed, in the early stages, round dull white spots, later, there was a semitranslucent grayish zone, with a depressed white center, rarely, a narrow purple zone of congestion might form around the lesion. The lung lesions were at first punctate hemorrhagic spots, but when fully developed appeared as glistening grayish nodules, hemorrhage being seen in the center. McIntosh and Scarff (1929) regarded the changes as those of an infective granuloma affecting primarily the smaller blood vessels, there being endothelial proliferation, tissue necrosis, and later, fibrosis. (For other papers on the pathology of generalized vaccinia see Ledingham, 1924; Ledingham and Barratt, 1929, Stewart and Duran-Reynals, 1929.)

Blood Changes in Infected Rabbits

It appears that workers have seldom reported on this question. Hine (1926) described the following changes in the blood of rabbits inoculated cutaneously with vaccinia. hemolysis, and the presence of bilirubin; an invasion of normoblasts, and a lowering of the red cell count and hemoglobin level, mainly from the 3rd to 8th day, a polynucleosis began about the 4th day, and lymphocytosis about the 6th to 7th day, both these reactions being over by the 11th day. Douglas, Smith, and Price (1929) described the following changes in 2 animals inoculated intravenously and developing generalized vaccinia: On the 2nd day after injection the number of red cells began to fall, and this continued till the 7th day, when the count was about half the normal Then the count increased, to become normal from the 15th to 16th day. The color index was raised during the fall. Normoblasts were found, especially shortly after injection. The total white count was reduced for 2 to 3 days, it then increased, to reach a maximum about the 9th day. The large mononuclears showed the greatest increase, polymorphs were increased to a lesser extent, while lymphocytes were definitely decreased, myelocytes were found from the start

Distribution of Virus in Infected Rabbits

Brain and cord As would be expected, virus is found in the brain, and sometimes the cord, after direct intracerebral injection. It probably disappears from the brain fairly rapidly (10 days—Gordon, *Report*, 1928, see also Herzberg, 1931 *a*); on occasions, virus appears to remain for a long period—Perdrau (*Report*, 1928), in fact, finding it 7 months afterwards Numerous authors have isolated virus from the brain, and less commonly the cord, of rabbits infected with vaccinia by routes other than intracerebral (Levaditi, Harvier, and Nicolau, 1921, Plotz, 1922, Blanc and Caminopetros, 1923, 1924 *b*, Levaditi and Nicolau, 1923 *a*, Bankine *et al.*, 1924; Huon and Placidi, 1924, Lucksch, 1927, Zurukzoglu, 1927; McIntosh and Blaxall, see *Report*, 1928, Berger, 1929, Douglas, Smith, and Price, 1929, Cattaneo, 1930) This localization of vaccinia has been noted after injection of both dermo- and neuro-viruses by the following routes dermal, subcutaneous, corneal, intratesticular, intravenous, and intrasciatic

It has been found that the intracerebral injection of human brain tissue serves to localize vaccinia in the brain of infected animals (Levaditi and Nicolau, 1923 *c*, Levaditi, Lépine, and Troisier, 1928)

Cerebrospinal fluid Gildemeister and Hilgers (1930 *a*) isolated virus from the cerebrospinal fluid in 5 out of 34 rabbits after skin inoculation

Blood. Vaccinia has been found in the blood of infected rabbits, although not very constantly (Ohtawara, 1922, Gildemeister and Heuer, 1927 *a*, 1928, McIntosh and Blaxall, see *Report*, 1928) It appears that an explanation for the failure of various workers to find the blood infective is now available, because Smith (1929) showed that virus present in blood, was rapidly removed by the blood cells In this way, whole blood appeared to be virus-free, but after fractionation virus was detected in the white cells He found vaccinia in the blood up to 8 days after in-

of vaccinia in monkeys after intracutaneous injection They found that the reaction appeared later than normal, was more necrotic, and healed more slowly. Extirpation of the spleen also caused retardation of the vaccinial process

The histological changes in the skin of the monkey infected with vaccinia are very similar to those produced by variola (see p. 308). Vesicular change occurs in the epidermis, and Guarnieri bodies can be found. Lesions are found in the dermis also, such as proliferation of the capillary endothelium and cellular infiltrations.

The Experimental Production of Encephalitis

A number of important papers has been published on the development of meningo-encephalitis in monkeys, following injection by intracerebral or other routes. This matter is of main interest in view of the claims that have been made for vaccinia virus as the etiological agent of postvaccinal encephalitis of man The papers will now be cited *seriatim*:

(*a*) *Levaditi, Nicolau, and Sanchis-Bayarri* (1927) found that monkeys developed meningo-encephalitis after intracerebral injection with neurovaccinia. Death usually occurred shortly after Histologically the lesions did not resemble those of postvaccinal encephalitis

[illegible]

corded changes in the central nervous system after dermal inoculation of a dermal strain of lymph. With large doses perivascular demyelination was noted.

(*d*) *Eckstein and Sarvan* (1930) failed to notice any nervous symptoms or to demonstrate histological changes in the brains of monkeys vaccinated intradermally and then subjected to certain procedures e g, chronic alcoholic intoxication, starvation, or splenectomy.

(*e*) *Hurst and Fairbrother* (1930) carried out experiments on the intracerebral injection of monkeys with a testicular strain of virus. They found that an early symptom of infection was nose-rubbing Then the animals became apathetic, and weakness developed on the contralateral side of the body, mainly the leg Jacksonian fits might occur, contralateral hemianopia and, later, complete blindness

[illegible]

granule cells. Enlargement of the glial cells was noted as early as the 3rd day. There was evidence of nerve cell degeneration, chiefly of the smaller cells, in the superficial layers, the changes had not, however, advanced to neuronophagia No evidence of demyelination, in any way comparable to that observed in human postvaccinal encephalitis, was seen

In addition, they carried out experiments in which monkeys were injected intradermally with ordinary vaccine lymph, or with neurovaccine; these animals were also injected intracerebrally with normal brain tissue, or intrathecally with horse serum, in an attempt to localize the virus None of these monkeys developed any lesions of consequence

(*f*) *Eckstein* (1931 *a, b*) showed that monkeys could be infected with vaccine lymph injected into the suboccipital space After 3 to 5 days the animals developed meningeal and encephalitic symptoms, and died between the 6th and 31st days (usually before the 10th) Histological examination showed evidences of a meningo-encephalomyelitis closely resembling human postvaccinal encephalitis

(*g*) *Eckstein* (1932 *b*) carried out experiments in which vaccine virus was injected intravenously, and the circulation of blood in the brain altered by the following maneuvers application of diathermy, unilateral ligature of the jugular vein,

in the meninges They found that numbers of rabbits became immune, due to an inapparent infection.

(*d*) Levaditi and Vaisman (1931) found that rabbits bearing experimental syphilomata might contract a spontaneous neurovaccinial infection when housed in the vicinity of infected rabbits.

(*e*) Bardach (1931) isolated a virus strain from a case of spontaneous vaccinial infection. This strain had the somewhat unusual property of producing pleurisy and peritonitis, with much exudate, in rabbits (1932 *a*). On intracerebral injection of mice a fatal meningo-encephalitis developed (1933).

(*f*) Pearce (1940) isolated vaccinia from the testes of 2 rabbits.

(*g*) *The Rockefeller Institute epizootics of rabbit-pox.* Perhaps the best known of all these types of outbreak occurred in the Rockefeller Institute, New York (*Greene, 1934 a, b, 1935 a, b, Rosahn and Hu, 1935; Hu, Rosahn, and Pearce, 1936,* Pearce, Rosahn, and Hu, 1936 *a, b*; Rosahn, Hu, and Pearce, 1936 *a, b, c*). Within a period of 2½ years the rabbit breeding colony at the Rockefeller Institute was visited by 3 separate epidemics of rabbit-pox. The disease was very fatal, especially in the younger stock; lymphadenitis, orchitis, a skin rash, and eye lesions were commonly found Focal necrotic changes occurred in the internal organs.

The disease was caused by the virus of rabbit-pox which has been studied in detail, and is probably closely related to that of vaccinia, especially neurovaccinia, although it cannot be held to be identical It is more potent than neurovaccinia and considerably more so than dermal culture virus. These differences between the 3 strains can be demonstrated by animal inoculation, serum neutralization tests, and cross inoculation tests.

The important question that arises out of this work is whether neurovaccinia is not in reality a rabbit-pox strain, or derived therefrom. Borrel (1936) has suggested that neurovaccinia may be rabbit-pox, but this suggestion has been strongly countered by Levaditi *et al* (1938), who claim that neurovaccinia is a biological *modification of dermal vaccinia* (see p. 311).

VACCINIAL INFECTION OF MONKEYS

Numerous authors have studied the effects of vaccinia virus on monkeys (e g, Copeman, 1893–4 *a*, Sternberg and Reed, 1895, de Haan, 1896, Roger and Weil, 1902, Brinckerhoff and Tyzzer, 1906, Teissier, Duvoir, and Stevenin, 1911; Levaditi *et al.*, 1927, Aldershoff *et al*, 1929, Clearkin, 1929, 1930, Eckstein and Sarvan, 1930, Hurst and Fairbrother, 1930, Eckstein, 1931 *a, b*, 1932 *b*, 1933, Rivers, Sprunt, and Berry, 1933, Torres and Teixeria, 1934 *a*, Torres, 1935–6).

Rhesus, cynomolgus, cercopithecus, and cebus monkeys have been most commonly used, and the chimpanzee has also been shown to be susceptible.

Routes of Inoculation

Monkeys can be readily infected with vaccinia virus by application to the scarified skin, by intradermal inoculation, and by intracerebral injection (*vide infra*). Keratitis follows corneal inoculation Lesions are produced in the nasal, oral, and buccal mucosae after local application Apes can be infected by intratracheal and intrapulmonary injection (Eckstein, 1933).

After inoculation of the skin the animal usually develops a local vaccinial lesion after 3 to 4 days By the 5th day the eruption is raised, surrounded by an areola, shows a ring of vesicles, and is crusted centrally. By the 8th day the central crust has extended to cover some of the vesicles, subcutaneous edema is well developed. Sometimes a few "daughter" vesicles may appear. By 10 to 12 days the lesion has quite healed The animal may develop a slight temperature, the lymph nodes are enlarged Sometimes a generalized rash may occur.

Eckstein and Sarvan (1930) studied the effect of starvation on the development

ternal organs (except the liver) contained virus up to 24 hours after death but not after 48 hours.

Pathology

Skin. Copeman and Mann (1899) carried out a detailed study of the histological features of the skin after vaccination of the calf, and their results will now be quoted The earliest change is a preliminary dilatation of the intercellular lymph channels Thereafter, changes occur in the different layers of the epidermis as follows

After 48 hours, the basal cells have increased from 13 μ–15 μ to 30 μ–35 μ in length, and the nuclei from 10 μ to 13 5 μ Some cells, however, shrink owing to compression The remaining cells undergo reticulating or "ballooning" change The "balloons" are formed by the epithelium breaking up into a number of pillars surrounded by lymph, later, many of these are torn across, so as to form bullae These bullae separate the superficial epithelial cells from the deeper layers overlying the dermis The tension tears across intercellular bridges and cells are liberated in groups of 1 to 10 In the larger groups some cells appear to thrive, but the remainder show atrophic or reticulating change.

Guarnieri bodies are found in the epithelial cells from an early stage At first measuring only about 0 5 μ they soon increase up to 2 μ.

The cells in the deeper layers show a special tendency to undergo nuclear proliferation. This presents itself as follows Large cells are formed in which reticulation begins around the nucleus and spreads peripherally. The cell loses its definite contours and leukocytes enter. The original nucleus shrivels and becomes eosinophilic. These cells form one type of vaccinial giant cell Another type of giant cell is characterized by the condensation of its cell body due to absorption of fluid Daughter cells then form within the mother cell. These 2 types of giant cell develop at the height of the reaction (from the 2nd to 4th day) Another type, however, develops among healthy cells, mainly on the 4th and 5th days, it is represented by numerous vesicular nuclei free in a cavity between the surrounding cells

In the dermis the earliest change is evident 48 hours after vaccination, as a necrosis of the basal membrane which swells and becomes strongly eosinophilic This change occurs beneath the vaccinated area, even distant from the point where the epidermis is scarified. The reaction is noted at a time when the epithelial changes are only slight in extent Edema and leukocytic infiltration begin on the 2nd day and are maximal by the 5th Numerous free granules, probably leukocytic, are to be found in the lymph spaces

Cornea Tyzzer (1904) described the changes as follows Naked-eye, it is difficult to see lesions in the vaccinated cornea, but when placed in Zenker's fluid several minute rounded opacities appear (one may also use fluorescein). Two days after inoculation, many Guarnieri bodies may be found in certain areas After 5 days there are small foci of epithelial cells showing vacuolar degeneration changes

Nasal mucosa Tyzzer (1904) described the following changes in the nasal septum inoculated with vaccinia The vaccinated area becomes opaque and elevated, and extends slowly day by day After 5 days large vesicles are found in the epithelium, due to vacuolar degeneration of the epithelial cells, and numerous Guarnieri bodies occur

VACCINIAL INFECTION OF OTHER ANIMALS

Guinea-pigs

Vaccinia virus can be transmitted to guinea-pigs (Levaditi and Nicolau, 1923 *a*, Bedson and Bland, 1928, Bland, 1928 *b*, Grinker, 1930–1, Herrmann, 1931, Munsterer, 1934–5, Stritar and Hudson, 1936, Kroemer, 1938)

Bland (1928 *a*, *b*) studied the reaction of the scarified and epilated skin to inocu-

or repeated suboccipital puncture. The monkeys developed a diffuse encephalitis, and in some cases a meningo-encephalomyelitis.

(*h*) *Eckstein* (1932 *b*) was unable to produce involvement of the central nervous system in monkeys by injection of virus into the brachial plexus.

(*i*) *Pette and Kornyey* (1933) were not able to produce changes resembling those of human postvaccinal encephalitis by injection of dermal vaccinia into the carotids or the cisterna magna.

(*j*) *Rivers, Sprunt, and Berry* (1933) have carried out experiments in an attempt to produce encephalitis in monkeys (1) Following intracranial injection, a severe meningitis and a mild encephalitis developed, but there was no evidence of perivascular demyelination (2) The injection of virus intracranially in immune and partially immune monkeys did not produce a disseminated encephalomyelitis. (3) Similar injections with the addition of Duran-Reynals' testicular factor (*vide infra*) did not produce encephalomyelitis either.

(*k*) *Eckstein* (1933) found thromboses, hyperemia, and glial proliferation in the brain following intratracheal or intrapulmonary injection

Summary. Some difference of opinion is manifested in the above work It may, however, be concluded that the majority has found that meningitis is the characteristic lesion produced by the intracranial injection of vaccinia. Further, after intradermal injection, lesions do not usually develop in the brain

Distribution of Virus in Infected Monkeys

It appears that virus spreads fairly widely throughout the body after injection For instance, virus was isolated from the brain 8 to 10 days after dermal inoculation with neurovaccinia (Levaditi and Nicolau, 1926). McIntosh and Blaxall (*Report*, 1928) vaccinated a monkey in the skin and were able to isolate virus from the brain, liver, spleen, and blood on the 8th day Following intracerebral inoculation of monkeys with testicular virus, Hurst and Fairbrother (1930) found virus in the brain, lumbar cord, spleen, liver, kidney, lung, adrenal, testis, and ovary

VACCINIAL INFECTION OF BOVINES

Calves are most readily infected by application of virus to the scarified skin, and by *intradermal injection. Calves, of course, are the animals in which vaccine* lymph is most frequently passaged for use in human vaccination and this matter is fully discussed later in the section on vaccination (see Ch XXXIV).

Calves can be infected by other routes than the skin, however. After corneal scarification a well-marked keratitis develops in 6 to 7 days, infection can also be secured by injection into the anterior chamber (Straus, Chambon, and Ménard, 1890). Calves can be infected on the nasal mucosa (Tyzzer, 1904) Noguchi (*1915*) found that vaccinia could be passaged in young bulls by intratesticular injection

Chaumier (1924) reported that the subcutaneous inoculation of calves with vaccinia produced large infiltrated and edematous areas that gave a profuse yield of virus

Distribution of Virus

Virus may diffuse throughout the body of bovines after inoculation of the skin. For example, it may occur in the spleen, lymph glands, kidneys, and brain, just before death, in heifers inoculated intradermally (Barikine *et al*, 1924), Blaker (1936) isolated virus from the amniotic fluid of cows inoculated cutaneously McIntosh and Blaxall (*Report*, 1928) vaccinated calves on the skin and found virus in the blood, liver, and brain for about a fortnight Zeller *et al.* (1933) also studied the distribution of virus in the bodies of vaccinated calves It was isolated from the blood on the 6th and 7th days, but not in the first 4 days. Virus could be isolated from the flesh immediately after death, but not 24 hours later The in-

THE PRIMARY TISSUE REACTION IN EXPERIMENTAL VACCINIA

Much work has been carried out on the pathology of vaccinia, and considerable reference to such papers has already been made. It falls now to link up some of the above with work which has been carried out in other directions, with a view to studying the primary reaction of the tissues to vaccinia virus.

1. *Copeman and Mann* (1899) studied the histology of the skin of the vaccinated calf and found that necrotic swelling of the basal membrane is the primary change This is soon followed by swelling of the epidermal cells.

2. *Ledingham* (1924) carried out important studies in this connection From a study of the pathology of the skin and certain other tissues, he concluded that vaccinia has no epiblastic affinity (see also p 314). He regarded the vaccinial lesion as an acute infective granuloma in which the reticulo-endothelial system is predominantly involved.

3. *Watanabe* (1924), studying the histology of generalized vaccinial lesions, found no evidence of epidermal reaction, there being only inflammatory foci in the subepithelial tissue.

4 *Amies* (1938) carried out experiments in which he studied the reaction of the skin to inoculation of polydisperse and homogeneous suspensions of elementary bodies (see also p. 315). After inoculation of polydisperse suspensions (crude lapine) there is early and marked infiltration of both dermis and epidermis with inflammatory cells When homogeneous suspensions (washed elementary bodies) are used, the infiltration by inflammatory cells is much reduced, there is marked proliferation and "ballooning" of the malpighian cells, inclusions are a conspicuous feature. He believed that this change in the nature of the lesions is produced by a process of adaptation and selection, whereby the normal ectodermal activities of the virus are intensified.

5 *Widelock* (1938) studied the early changes in the skin after intracutaneous injection of vaccinia There is a correlation between the multiplication of the virus and the increase in the size and numbers of the epithelial cells. The earliest change is an increase in the size of the epithelial cells, later they increase also in numbers The increase in the total width of the epithelium is greatest when the virus attains its maximum multiplication. He considered that cellular infiltration of the epithelium occurs secondarily to the epithelial cell changes, which he regarded as primary.

In confirming work of Ledingham (1926 *b*), he showed that the injection of a mixture of India ink and virus prevents the formation of a typical vaccinial reaction, the virus, however, still multiplies and epithelium still undergoes proliferation.

6. *Observations in the egg* The infected chorio-allantoic membrane forms an ideal site for the study of the histological reaction to vaccinia Most workers have noted that the main reaction concerns the epithelial cells, Guarnieri body production often being an early feature (see p. 343) There is usually also considerable involvement of the mesoderm and, to a lesser extent, the endoderm Buddingh (1936 *a*) noted that a strain of neurotesticular virus had a particular predilection for the mesoderm

Levaditi and Voet (1937) have studied the action of both dermo- and neuroviruses on the chorio-allantois The first change, evident from the 18th hour onward, is an ectodermal thickening, these cells show numerous mitotic figures, are swollen, and contain both elementary and inclusion bodies. After 25 hours, inflammatory changes are seen in the mesoderm. From this time onward the ectoderm undergoes marked proliferation, and cell-nests form in the mesoderm From these studies they concluded that the vaccinia virus has a dominant epithelial affinity.

Conclusions It seems unwise to adopt too dogmatic an attitude with regard to the primary tissue reaction to vaccinia There is no doubt that the epidermal cells

lation of vaccinia; after the virus had been passaged for some generations, it acquired the property of producing pustular lesions. Injections may also be administered into the skin of the guinea-pig's pad.

After intracerebral injection of neurovaccinia, guinea-pigs die from meningo-encephalitis. There are mononuclear and polymorph cells in the meninges; the brain shows the changes of acute encephalitis, with inflammatory foci, and perivascular cuffing (Levaditi and Nicolau, 1923 *a*). Grinker (1930–1) stated that these animals can be infected by intracerebral injection of calf lymph, with the production, in some cases, of a meningo-encephalitis Olitsky and Long (1928), however, reported that neurovaccinia did not induce encephalitis in guinea-pigs on intracutaneous, intratesticular, corneal, or intracerebral injection.

Stritar and Hudson (1936) propagated virus by intracerebral injection of fetal guinea-pigs. Skin pocks were found, and necrotic areas in the lungs and kidneys

Mice

A number of authors has infected mice (Levaditi and Nicolau, 1923 *a*, Rosenau and Andervont, 1931; Buckup, 1935, Kanazawa, 1936; Eyer, 1937; Nelson, 1938, Bronson and Parker, 1941, 1944). They can be injected intracerebrally, when symptoms of encephalitis develop. Buckup (1935) found that strains of variola-vaccinia differed considerably in their powers of inducing meningo-encephalitis on intracerebral injection of white mice. Haagen (1934) found that by repeated brain passage a "fixed" strain could be produced, which caused death in 5 to 9 days. Mice can also be infected by application to the scarified skin, or by intraperitoneal, intravenous, or subcutaneous injection. Nelson (1938) found that after nasal instillation of culture virus, or dipping the nares in virus-containing liquid, the animals develop respiratory symptoms, and may die of pneumonia. The lungs show areas of consolidation, at first patchy, but later more confluent Histological examination shows necrosis of the bronchi, the alveolar walls are often necrotic, and the alveoli contain coagulated fluid and fibrin. Lister (1940) could not infect mice with Lister Institute lapinia.

Sheep

In certain institutes sheep are used to yield vaccine for human use, the virus being readily passed by dermal inoculation (Kasai, 1931)

Other Animals

Horses can be infected with vaccinia, and following intravenous injection generalized vaccinia may develop (Chauveau, 1865–6, 1877; also Raynaud, 1878).

Various authors have studied the vaccinial infection of the *chick* (Blanc and Caminopetros, 1923, Levaditi and Nicolau, 1923 *a*, Lowenthal *et al.*, 1925, Andervont, 1926 *a*, *b*, 1927, Findlay, 1927–8, Woodruff, 1930) Chickens can be infected by scarification of the comb or cornea, or cerebrally.

Chaumier (1924) reported that the subcutaneous inoculation of *asses* with vaccinia produces large infiltrated edematous areas that give a profuse yield of virus. [illegible] (Blaker, 1936) The *cat* can be infected cere- [illegible] [illegible] [illegible], especially on corneal inoculation (Findlay, 1927–8). After cutaneous inoculation with neurovaccinia a papulopustular eruption develops by the 4th day (Levaditi and Nicolau, 1923 *a*) Lesions can be produced on dermal inoculation, after treatment with thallium (Buschke *et al.*, 1931). It is also susceptible to intracerebral injection (Kubota, 1938).

The *ground squirrel* can be infected with vaccinia by corneal inoculation (Goschanskaja and Stschastny, 1933)

rabies did not develop, but, if vaccinia was injected shortly afterward, then rabies developed.

B. Poliomyelitis

Thompson (1930*a*) failed to adapt poliomyelitis virus to rabbits by admixture with vaccinia. Hurst and Fairbrother (1931) injected monkeys intracranially with a mixture of neurovaccinia and poliomyelitis virus. They did not find that the vaccinia modified the lesions of poliomyelitis, nor could it activate a weak strain of virus.

C. Foot-and-mouth Disease

Gildemeister and Helm (1932) recorded a definite antagonistic effect when mixtures of vaccinia and foot-and-mouth viruses were inoculated into guinea-pigs.

D. Herpes

A number of experiments has been carried out with herpes virus, because many used to believe that postvaccinal encephalitis was caused by the activation of this virus lying latent in the body, by the process of vaccination On the one hand, 2 groups of authors have adduced experimental evidence that vaccinia may have an activating effect on herpes virus Thus, when herpes virus was instilled intranasally, and at the same time neurovaccinia inoculated dermally, the animal often died from herpetic encephalitis (Levaditi and Nicolau, 1926) Further, Zurukzoglu *(1927) found that when either vaccine virus was injected intravenously, or herpes* subcutaneously, alone, then the rabbits lived. If the injections were combined the animals died from herpetic encephalitis On the other hand, the bulk of evidence appears to deny any activating role to vaccinia·

(*a*) For instance, in the *Report* of 1928, Gordon recorded the following experiments First, he inoculated rabbits intranasally with a nonencephalitogenic herpes strain, and simultaneously administered vaccinia nasally, or subcutaneously, no encephalitis developed. Secondly, he inoculated half a minimum lethal dose of

1. A sublethal dose of herpes was inoculated intracerebrally, 3 weeks later a cutaneous inoculation with vaccinia was performed
2. Neurovaccinia was inoculated nasally, and followed in 3 weeks by herpes intradermally
3. Herpes was administered nasally, and 3 weeks afterward a cutaneous inoculation with vaccinia was carried out
4. These procedures were, on occasions, followed by an intravenous injection of hexamine to endeavor to lower the blood-brain barrier, and to allow herpes to enter the central nervous system
5. Vaccinia (calf lymph) was inoculated cutaneously, and 5 days later herpes was *injected into the blood stream*

In none of these 5 experiments did any encephalitis result

(*c*) Perdrau (1928) studied the effect of inoculating a mixture of herpes and neurovaccinia intracerebrally in rabbits He found that a normal animal died in 2½ days, a rabbit immune to both viruses recovered, a rabbit immune to vaccinia died from herpetic encephalitis in 5 days, finally, a rabbit immune to herpes died in 5 days from vaccinia

(*d*) Berger (1929) inoculated rabbits on the cornea with herpes, *and later with* vaccinia, but no encephalitis developed

(*e*) Olitsky and Long (1928) could find no evidence that injections of neurovaccinia given corneally, by scarification, intratesticularly, or intracerebrally, stimu-

are often primarily and predominantly involved, but the other layers are not spared, and sometimes the mesodermal reaction in particular may be severe.

THE EFFECT OF CERTAIN BIOLOGICAL AGENTS ON THE VIRULENCE OF VACCINIA

Duran-Reynals's factor. Duran-Reynals (1929) reported that extracts of normal testis exalt the virulence of dermo- and neuro-viruses. The enhancing effect operates rather on the host cells than on the virus, for it was shown that after intravenous inoculation, virus localizes most readily in areas previously inoculated with testis extract. Moreover, enhanced lesions result if virus is inoculated into the skin up to 3 days after injection of extract. McClean (1930) showed that the intravenous injection of extract enhanced the lesions produced on intradermal inoculation About 25 per cent. of rabbits with enhanced lesions die from generalized vaccinia Kidney, brain, and liver extracts only show this enhancing power to a much less degree. The intradermal injection of fluid from embryo tissue and chicken sarcoma cultures secures the localization of intravenously injected virus; when virus is rendered almost inactive by the action of chloroform, potency can be restored by the addition of these agents, or of kieselguhr (Duran-Reynals, 1928 *a*).

[illegible] ct of testis extract should be made use [illegible] virus, the test material should be mix [illegible] experimental animals (Andersen, 1937 *a*). *Fellowes and Hudson (1939) have shown that the Duran-Reynals factor* occurs in both fetal and adult guinea-pig tissues.

Gastinel, Fasquelle, and Arnaud (1946) showed that there is no enhancement if the spreading factor and virus are inoculated together by scarification. On intradermal injection, the enhancing effect is noted slightly with dermal virus, but very markedly with neurovirus

Other enhancing substances Azoprotein solutions increase the size of dermal lesions (Claude, 1939), and a similar effect is secured by mucin (Clemmesen and Anderson, 1942) Sprunt (1942 *b*) concludes that if the interstitial fluid is decreased, *the lesions are more numerous, as virus is enabled to spread* Nontoxic rattlesnake venom exerts an enhancing effect (Duran-Reynals, 1939).

Gratia and Linz's (1931 a) phenomenon. These authors injected rabbits with testicular virus on the skin or intratesticularly, a day or so later a filtrate of a culture of *B coli* was administered intravenously A very severe hemorrhagic reaction followed in the inoculated testis, and in the lymph glands draining the site of dermal inoculation They also studied the effect of intracerebral injection of virus followed by *B coli* filtrate intravenously, some animals developed hemorrhages in the intestine, diaphragm, heart, lung, and lymph glands (Gratia and Linz, 1931 *b*)

Virulence-inhibiting effects Duran-Reynals and Stewart (1931) found that human sarcoma extracts inhibited vaccinial reactions Methionine and choline inhibit the dermal response (Sprunt, 1942 *a*), and a similar inhibition occurs in rabbits deprived of food (1942 *b*)

Rabbits inoculated with estrogenic hormones show an increased resistance, owing to increased tissue fluid and localization of the virus (Sprunt *et al*, 1938, Sprunt, 1941 *a*, *b*, *Taylor and Sprunt, 1943*)

SIMULTANEOUS INFECTION WITH VACCINIA AND OTHER VIRUSES

A. Rabies

Levaditi and Nicolau (1925) injected rabbits suffering from a latent neurovaccinial infection with street virus intracerebrally. Passage of brain tissue was carried out, at first rabies virus was demonstrable, but later vaccinia was found If rabies and vaccinia viruses were inoculated on the skin or cornea simultaneously,

rabies did not develop, but, if vaccinia was injected shortly afterward, then rabies developed.

B. Poliomyelitis

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3. Herpes was administered nasally, and 3 weeks afterward a cutaneous inoculation with vaccinia was carried out.
4. These procedures were, on occasions, followed by an intravenous injection of hexamine to endeavor to lower the blood-brain barrier, and to allow herpes to enter the central nervous system
5. Vaccinia (calf lymph) was inoculated cutaneously, and 5 days later herpes was injected into the blood stream

In none of these 5 experiments did any encephalitis result

(*c*) Perdrau (1928) studied the effect of inoculating a mixture of herpes and neurovaccinia intracerebrally in rabbits He found that a normal animal died in 2½ days, a rabbit immune to both viruses recovered, a rabbit immune to vaccinia died from herpetic encephalitis in 5 days, finally, a rabbit immune to herpes died in 5 days from vaccinia

(*d*) Berger (1929) inoculated rabbits on the cornea with herpes, and later with

lated Levaditi's C strain of herpes to produce encephalitis on intracerebral injection.

(*f*) *The cytoplasmic inclusions of vaccinia and the nuclear inclusions of B virus* or herpes virus have been found in single cells of the rabbit cornea inoculated with mixtures (Syverton and Berry, 1947 *b*).

RELATIONSHIP OF VACCINIA TO CERTAIN OTHER VIRUSES

A. Foot-and-mouth Disease

Uhlenhuth and Trautwein (1934–5) were not able to render guinea-pigs resistant to vaccinia virus by injections of foot-and-mouth virus (all 3 types were used). Conversely, injections of vaccinia did not protect the animals against foot-and-mouth disease.

B. Herpes Febrilis

Zurukzoglu (1927) claimed that vaccinial infection gave rise to some immunity to herpes, and vice versa. Gildemeister and Herzberg (1925) also claimed a relationship between herpes and vaccinia on the basis of cross immunity tests carried out *in vivo* and *in vitro*. Later (1927) they found that the majority of variola-immune rabbits only developed mild reactions on injection of herpes Many workers, however, have shown that there is no immunological relationship (Bedson and Bland, 1928, Perdrau, see *Report*, 1928; Keller and Schaefer, 1929, Gildemeister and Ahlfeld, 1937; Kanazawa, 1938).

C. Fowl-pox

Although Toyoda (1924) and Pandit (1926–7) claimed that fowl-pox could be converted into a virus resembling vaccinia by passage through experimental animals, it is not generally believed that the viruses of fowl-pox and vaccinia are related. Andervont (1926 *b*) could not demonstrate any resistance to fowl-pox in vaccinia-immune birds, nor was vaccinia-immune serum virucidal for fowl-pox virus Ledingham (1926 *a*) was unable to demonstrate any cross immunity Findlay (1927–8) and Woodruff (1930) were likewise unable to find any relationship.

D. Ectromelia

Vaccinia and ectromelia have similar hemagglutinating properties (see Ch XXXI) Specific antisera show some cross neutralization in the CCA-inhibition test, and immunized rabbits and mice show some degree of cross resistance It is considered probable that the 2 agents have a similar biological origin, and that ectromelia is a member of the animal pock disease group (Burnet and Boake, 1946)

CERTAIN MISCELLANEOUS EXPERIMENTS WITH VACCINIA

Pearce (1928 *a, b, c*) studied the effect of simultaneous infection with vaccinia and syphilis in rabbits, with the following results If vaccinia was injected intracutaneously, and at the same time *Treponema pallidum* intratesticularly or intradermally, then the syphilitic reaction was extremely severe A similar modification occurred if rabbits were injected intratesticularly with the 2 agents If the animal injected with *Treponema pallidum* was immune to vaccinia, this had the effect of causing the resultant syphilitic infection to be very mild.

It has been claimed that the injection of calf lymph or neurotropic virus cerebrally in mice has some protective effect against death from the cerebral injection of *H pertussis* at a later date (Dalldorf, Cohen, and Coffey, 1947)

Neurovaccinia survives if injected into epitheliomata, but not sarcomata, of rats and mice, if the injection is intravenous, virus localizes in epitheliomata, but not sarcomata (Levaditi and Nicolau, 1923 *b*). These workers also studied the immunity reactions of vaccinial infection of tumors, epitheliomata growing in a vac-

cinia-immune animal participated in the general refractory state, but if this tumor was grafted to a susceptible host it was no longer refractory to injection of virus

Rivers and Pearce (1925) studied the effect of inoculating a transplantable rabbit tumor with vaccinia. It was found that the virus survived in the tumor for at least 64 days, and was carried on when the tumor was passaged. The animals bearing these tumors infected with virus became immune to vaccinia, but this did not destroy the virus

Levaditi and Schoen (1936) inoculated rabbits bearing Shope's papillomata with neurovaccinia either intravenously, intradermally, or into the tumor itself. On the 5th day virus was found in the tumor in every case. Syverton and Berry also found that these papillomata could be infected by vaccinia (1947 *a*).

with vaccinia when in-

12 days and can cause

CHAPTER XXIX

THE VARIOLA-VACCINIA VIRUS: ELEMENTARY AND INCLUSION BODIES

1. THE ELEMENTARY BODIES OF VARIOLA-VACCINIA

For many years it has been known that small granules can be demonstrated in various suspensions prepared from tissues infected with vaccinia. Although the credit of discovering these bodies is usually given to Paschen (1906)[1] they were actually discovered many years earlier by Buist (1886) in Edinburgh (see Gordon, 1937, Mackie and van Rooyen, 1937). These bodies are known variously as elementary bodies, EBs, and Paschen bodies, although it would seem to be more accurate to call them Buist bodies. Mackie and van Rooyen have proposed the term *Buistia pascheni*, thereby commemorating the work of both Buist and Paschen Various other names have been proposed too for instance, Aragão (1933) suggested that the name *Chlamydozoa ribasi* be applied to the elementary bodies of alastrim, and von Prowazek employed the term *Chlamydozoa variolae*. Goodpasture (1933) introduced the designation *Borreliota variolae hominis* to describe the elementary bodies of smallpox, and *Borreliota variolae bovis* for those of vaccinia.

So many authors have described the occurrence of elementary bodies in vaccinial, alastrinic, and variolar lesions of man and animals that only comparatively few can be mentioned by name (von Prowazek and Aragão, 1909, Aragão, 1911, MacCallum and Oppenheimer, 1922, Craciun and Oppenheimer, 1926, Mudd and Nakajima, 1929–30, Eagles and Ledingham, 1932; Goschanskaja and Skrotzky, 1932, Paschen, 1933–4, Taniguchi *et al*, 1932 *a*, *b*, 1933, 1934, Craigie, 1933, Haagen and Kodama, 1934, Eagles, 1935 *a*, Haagen, 1935, Parker and Rivers, 1935, Turevich, 1935, Kanazawa, 1936) Smadel and Hoagland (1942) have published a general review on the properties of vaccinial EB's

Morphology and Size of Elementary Bodies

The elementary bodies of vaccinia or variola can be demonstrated microscopically in films stained by Paschen's or other method, as small granules, sometimes occurring in pairs, short chains, or clusters (see Figs. 4 and 5) It appears from experimental observations that elementary bodies enter cells and proliferate to form inclusion (Guarnieri) bodies. Examination of these inclusion bodies shows that they are, at any rate partly, composed of elementary-body-like granules

The size of elementary bodies can be estimated by various direct micrometric methods (see Ch I), when they are found to measure approximately 0 2 μ in diameter Of recent years, more accurate methods have been introduced, following certain earlier experiments by Yaoi and Kasai (1929 *e*) By ultrafiltration for example, the following results have been obtained 0 125 μ to 0 175 μ (Elford and Andrewes, 1932), 0 14 μ to 0 16 μ (Levaditi, Paic, and Krassnoff, 1936, see also Paic *et al*, 1938)

Ultracentrifugation studies have also been used to determine the size of vaccinial elementary bodies (Bechhold and Schlesinger, 1931, Elford and Andrewes, 1936, Gratia and Plotz, 1938, Pickels and Smadel, 1938) The following results have been obtained 0 17 to 0 18 μ (Elford and Andrewes), 0 21 to 0 23 μ (Bechhold and Schlesinger), 0 236 μ (Pickels and Smadel)

[1] References are appended at the conclusion of Ch XXXV, p 403 et seq

Elementary bodies give a negative reaction in Feulgen's test (Haagen, 1937 *b*, Robinow and Bland, 1938).

Electron micrographs show vaccinial EB's to be brick-shaped, with areas of electronic density, there appears to be a limiting membrane (Greene, Anderson, and Smadel, 1942; Smadel, Anderson, and Green, 1942, Groupé, Oskay, and Rake, 1946, Sharp *et al.*, 1946, Dawson and McFarlane, 1948, Ruska and Kausche, 1943, see also Fig. 29A). Nagler and Rake (1948) estimate the size at 244 × 302 m μ.

The Sites of Occurrence of Elementary Bodies

Paschen bodies have been found in all types of vaccinial infection, and in cases of variola and alastrim The sites in which they can be demonstrated most readily are as follows. in human vaccinia, variola, or alastrim vesicle fluid, in vaccinial dermal pulp obtained from calves, rabbits, and other animals, in testicular suspensions of infected rabbits, in exudate after nasal instillation of mice (Nelson, 1938). Certain investigators have demonstrated the occurrence of elementary bodies in stained smears of infected tissue cultures (Haagen, 1931, Nauck and Paschen, 1932 *a*, *b*, Taniguchi *et al*, 1933, Haagen and Kodama, 1934, Togounova, 1935, *et al*, 1935, Nauck and Robinow, 1935–6). Elementary bodies can be demonstrated very readily by suitable staining in smears of infected chorio-allantoic membranes (Goodpasture *et al*, 1932, 1935, Nauck and Paschen, 1933, Goodpasture and Buddingh, 1934, Peragallo, 1936–7, Lazarus *et al*, 1937, Stevenson and Butler, 1939).

The Preparation of Elementary Body Suspensions

Following certain earlier experiments (MacCallum and Oppenheimer, 1922, Craciun and Oppenheimer, 1926), it is now possible to obtain EB suspensions freed almost completely from extraneous material. Satisfactory EB preparations can be obtained only by using active, recently passed, material as seed (Beard *et al*, 1937, Amies, 1938). Many methods have been used for making suspensions of vaccinial EB's, for example

1 *Craigie's method* is described in Ch XI

2. *Smadel and Wall's (1937) method* Up to 25 chorio-allantoic membranes are rinsed in Locke's solution They are then dropped singly into ethyl ether, mixed in Locke's solution, and ground, 10–20 c c of dilute buffer (pH 7 2) is added, and the suspension shaken The sediment is deposited at 1,000 r.p m , resuspended in buffer and recentrifuged Both supernatants are pooled and spun at 1,000 r p m for 10 minutes The supernatant is then run in the Swedish angle centrifuge for 1 hour at 3,500 r p m The sediment, which contains the EB's as well as debris, is taken up in 5–10 c c. buffer (pH 8 0), and 0 5 c c filtered 1 per cent commercial trypsin (freed from lipoids by extraction with ether) is added The mixture is incubated at 37° C for ½ to 1 hour The digested material is then washed 3–7 times, using the angle centrifuge After the final washing, the suspension is spun in the horizontal centrifuge for ½ hour at 2,500 r p m to remove particles larger than the EB's Levaditi and Giuntini (1940) and McIntosh and Selbie (1940) have also used trypsin

3 *Henderson and McClean (1939)* used the method introduced by *Behrens and Nielsen (1935)*. Ground vaccine pulp is made ... ing, coarse tissue particles are remov... precipitated with citric acid, or dialyz... performed at 2,000 r p m for ½ hou... sodium carbonate, and 0 43 per cent NaCl and 1 per cent Difco proteose peptone are added These preparations can be kept for many weeks To keep for prolonged periods, filter the supernatant through Berkefeld V and Gradocol filters, desiccate from the frozen state in 1 c c amounts in ampules, and seal in nitrogen

4 *Ledingham's (1931) method* Early material from the skin is thoroughly triturated in a mortar with distilled water The crude suspension is then shaken up in a flask with at least an equal volume of ether and allowed to stand on the bench for at least 24 hours, being frequently shaken up during this period The suspension is then centrifuged at high

speed. The upper layer of ether, and the large middle layer of light fatty matter which rises to the surface are removed, leaving an underlying opalescent fluid. This underlying layer, together with the deposit, is again centrifuged at high speed for at least 1 hour (9,000 r.p.m.). The resulting deposit is collected after discarding the supernatant. This small deposit, consisting of EB's and cell debris is suspended in formol saline (1/400). By further fractional centrifugation, it is possible to eliminate practically all grosser debris, leaving EB's only.

The Infectivity of Elementary Bodies

It can be shown readily that the infectivity of vaccinia virus is intimately connected with the elementary bodies. For instance, if suspensions of elementary bodies are centrifuged, tests show infectivity to be associated with the elementary body deposit, and the supernatant fluid to be noninfective (Eagles and Ledingham, 1932). Vaccinial infection can be transmitted in series by means of washed elementary bodies (Craigie, 1933). When suspensions are passed through a Seitz filter, the material which remains behind on the disk contains the elementary bodies. By suitable washing and centrifuging, it can be shown that a suspension of the elementary bodies has a similar virus content to that of the original extract (Craigie, 1932 *a*).

Certain methods have been described whereby the numbers of elementary bodies in any fluid may be estimated. Such studies have usually been concerned with associating the number of elementary bodies with the degree of infectivity of the fluid containing them. Parker and Rivers (1936 *b*), for instance, have worked in this connection. They found that elementary bodies actually could be counted, by replicate counts, using a Petroff-Hauser chamber. Further, they found that the elementary body content of a suspension could be determined by using Gates's densitometer. They attempted to correlate the numbers of elementary bodies and infective units in any given suspension. Rabbits were infected with virus intradermally and the end point of the titration was taken as the dilution of virus which would lead, theoretically, to an equal number of positive and negative results. Using these methods, Parker and Rivers found that there was a direct correlation between the numbers of elementary bodies and the number of infective units in any given suspension.

Parker (1938 *b*) has described a method whereby the number of virus particles needed to cause infection of the rabbit's skin can be estimated. Intradermal injections of diluted suspensions of virus are made in rabbits. The percentage of inoculations of each dilution giving lesions is then noted, and statistical analysis applied. Parker studied various strains of virus by this means, and found that infection followed inoculation of a single particle of the New York City Board of Health strain, of a tissue culture derivative of this strain, and of the Noguchi strain. However, the tissue culture strain produced a very much less severe reaction than did the New York strain. He concluded, therefore, that factors other than pure infectivity must be responsible for the different reactions observed.

A number of later workers has investigated this problem, and their work serves to emphasize the complexity of the position. It appears that though theoretically certain strains may be so infective that a single EB can cause infection, in practice a minimal infective dose usually contains several EB's. Estimates of the number of EB's per minimal infective dose have been made, for example 4.2 was the average figure obtained by Smadel, Rivers, and Pickels (1939). Sprunt, Marx, and Beard (1940) found that an average of 366 bodies behaved as an infectious unit. They agreed that under ideal conditions a single EB might behave as an infective unit. The frequency of positive and negative results on the rabbit skin followed the curve to be expected if a single EB is infective. Discrepancy was dependent on the experimental conditions with respect to the strain of EB, the volume of the inoculum, and the variation in host susceptibility.

Levaditi and his coworkers have published many papers on this subject. They worked chiefly with EB suspensions prepared from chorio-allantoic membranes by trypsinization and centrifugation They counted the number of EB's by fluorescence microscopy in a counting chamber, and determined the number of EB's per minimal infective dose, usually by intradermal or cerebral injection in rabbits (Levaditi, 1939 *a, b, c*, 1941 *a*, Levaditi *et al*, 1939 *a, c*, 1940 *a, b*, Lépine, Levaditi, and Giuntini, 1944). They estimated the number of EB's of neurovaccinia in a minimal infective dose, as shown by cerebral titration, as between 21 and 390 (1939 *c*). They also showed that if numbers of elementary bodies too few to produce a macroscopic lesion in the skin were injected, multiple microscopic foci could be found, strongly suggesting that the minimal infective dose judged by naked eye examination contains many EB's (Levaditi *et al*, 1940 *a*)

The main factors that determine the infectivity of EB suspensions may be summarized as follows·

1. The EB is probably possessed of an inherent virulence depending on the strain. Parker, Bronson, and Green (1941) titrated 8 different strains of vaccinia in various animals They concluded that in a virus of maximum virulence an infectious unit was present in a single EB, but in strains of lower virulence the infectious unit contained many EB's.

2. The source of the suspension is of importance. For example, the number of EB's needed to produce a dermal lesion is much smaller if the suspension has been prepared from the chorio-allantois rather than the skin (Levaditi *et al*, 1940 *a*, Levaditi and Reinié, 1939 *a*).

3. The number of infectious units found in a given suspension depends chiefly on the concentration of EB's and not on the total volume. Smaller inocula are relatively more effective than larger (Sprunt, 1939, 1941 *a*).

4 The species of animal used for the test inoculation is of importance, rabbits, for example, being more sensitive than monkeys (Levaditi *et al*, 1940 *a*) Different results will be obtained according to whether the inoculation is made cerebrally or dermally (Levaditi and Reinié, 1939 *a*).

5 The question of variation in the susceptibility of the host is fundamental (Bryan and Beard, 1940 *a, b*, Sprunt and McDerman, 1940, Sprunt, Marx, and
B ways, e g, by giving estro-
g the amount of intercellular
fl (1941 *a*) concluded that in
addition
number
of host c
virus, e
of a lesion Spreading virus over a larger area increases the probability of a lesion The ratio of virus particles to host cells partly determines whether a lesion occurs.

Immunological Reactions of Elementary Bodies

This question is fully discussed in the chapter on the immunological reactions of variola-vaccinia virus (Ch. XXXIII), but here it may be said that elementary

standing

Chemical Composition of Elementary Bodies

It has been found that washed elementary bodies contain ash, carbohydrate, lipoid, fat, and nitrogen (Hughes, Parker, and Rivers, 1935, Moriyama 1937 *b, c, d;* Moriyama and Ohashi, 1939).

Hoagland, Smadel, and Rivers (1940) gave the following figures total nitrogen, 15.3 per cent., total carbon, 33.7 per cent., total phosphorus, 0 57 per cent.; cholesterol, 1.4 per cent.; phospholipoid 2.2 per cent.; neutral fat, 2 2 per cent, reducing sugars after hydrolysis, 2.8 per cent.; cystine, 1 9 per cent

Nucleic acid has been found, mostly consisting of the thymus type (Hoagland *et al*, 1940 *a*, Smadel, Lavin, and Dubos, 1940). Biotin has been demonstrated (Hoagland *et al.*, 1940 *c*), also copper (Hoagland *et al.*, 1941 *a*).

The fluorescence exhibited by purified EB's in UVL is due to flavin-adenine-dinucleotide (Hoagland *et al*, 1941 *b*).

EB's contain a lipoid material probably derived from the host, and adsorbed onto the virus, after extraction with ether or benzene, infectivity is unimpaired (McFarlane and Macfarlane, 1939, McFarlane *et al*, 1939). The virus particle has large amounts (7–8 volumes) of water associated with it in the form of an atmosphere (McFarlane *et al.*, 1939).

Metabolism of Elementary Bodies

Breinl and G'owazky (1935) found that there was evidence of metabolism in aerobic tests, but that this was very feeble in anaerobic tests Parker and Smythe (1937) found that the quantities of oxygen consumed, and acid liberated, were very small, further, the great part of the gaseous exchange took place during the first hour of test There was no increased absorption of oxygen on addition of glucose The virus was found to be perfectly active at the end of the experiment. They maintained that the results were compatible with a survival, but not with growth, of virus.

EB suspensions show phosphatase, catalase, and lipase activity (Macfarlane and Salaman, 1938, Hoagland *et al*, 1942) Suspensions show no dehydrogenase activity toward a number of substrates (Macfarlane and Salaman, 1938) In later work, it was shown that suspensions lacked zymohexase, enolase, α and β glucosidases, and nucleosidase, they hydrolyzed ribonucleic acid (Macfarlane and Dolby, 1940)

The Reaction of Elementary Bodies to Physical and Chemical Agents

The influence of reaction has been studied (Beard *et al*, 1937, 1938, Moriyama, 1937 *b*). Infectivity is rapidly lost at pH4 and 10, but is stable in between. The isoelectric point is 4 3–4 6

Sonic and supersonic waves do not appear to disrupt EB's (Rivers, Smadel, and Chambers, 1937; Hopwood, Salaman, and McFarlane, 1939).

X-rays cause inactivation (Gowen and Lucas, 1939) Lea and Salaman (1942), using alpha, x-, and gamma rays found that the virus could be inactivated by a single ionization They calculated that the radiosensitive material represents an area of about ¼ of the cross sectional area of the EB, but only 1/200 of its volume The radiosensitive targets, they suggested, may be genes Bonet-Maury (1941) and Bonet-Maury and Pérault (1941) have studied the action of the alpha rays of radon Irradiation with *ultraviolet light* destroys virus in suspensions or atomized form (Giuntini and Reimé, 1939, Levaditi *et al.*, 1939 *b*, Giuntini, 1941, Edward, Lush, and Bourdillon, 1943)

As regards *storage*, EB's can be maintained in an infective condition by desiccating *in vacuo* from the frozen state Even in broth they retain activity for considerable periods (Amies, 1934 *a*) Suspensions are rendered more stable by the addition of 0 1 per cent gelatin or 1 per cent peptone (Behrens and Ferguson, 1935)

Various *chemicals* inactivate EB's, e g, formol (Craigie and Wishart, 1933), 50 per cent or 75 per cent ethyl alcohol or acetone inactivate EB's in 3 hours at 14° C. (McClean, 1945) Pepsin and papain cause solution, with the release of amino nitrogen, crystalline trypsin and certain other enzymes are without effect (Hoagland *et al.*, 1940 *b*).

Certain Physical Properties

The density of EB's lies between 1.12 and 1.18 (MacCallum and Oppenheimer, 1922; Elford and Andrewes, 1936, McFarlane, 1938, Smadel *et al*, 1938, Moriyama and Ohashi, 1939). The weight of a single EB has been estimated at 5.2 to 5.34 $\times 10^{-15}$ gm (Smadel, Rivers, and Pickels, 1939, McIntosh and Selbie, 1940)

Virus can be aerosolized and recovered from the atmosphere (by exposure of the chorio-allantois) as late as the 8th hour after spraying (Buchbinder and Solotorovsky, 1941).

2. THE INCLUSION BODIES OF VARIOLA-VACCINIA

The large eosinophilic inclusion bodies of variola-vaccinia are among the best known of all inclusions, and are held to be quite specific Although it is possible that these bodies may have been described by Weigert in 1874 and by Pfeiffer in 1887, the main credit goes to Guarnieri (1892, 1894 *a*, *b*, 1897), the inclusions being universally known by his name. Although demonstrable in human tissue from cases of smallpox, Guarnieri bodies have been most studied in experimental animals, particularly in the rabbit's cornea, and more recently in infected eggs and tissue cultures

A very large number of authors has confirmed Guarnieri's original findings, and certain of these may be specially mentioned (Babès, 1894, Clarke, 1894, 1895, Monti, 1894 *a*, *b*, Pfeiffer, 1894, 1895, Ruffer, 1894, Ruffer and Plimmer, 1894, von

The Morphology and Occurrence of Guarnieri Bodies

Fully developed Guarnieri bodies are comparatively large (up to 10 μ), and usually circular or ovoid structures While the inclusions of vaccinia and variola are usually eosinophilic, those of alastrim are basophilic, these and certain other differential features are more fully described below Although usually homogeneous, Guarnieri bodies may appear definitely granular even in ordinary stained films, and by special modern methods of examination it has been shown that Guarnieri bodies are probably composed of masses of elementary bodies held together in a matrix They may be single or multiple, and are often surrounded by an unstained halo They are usually found in the perinuclear space.

Guarnieri bodies can be demonstrated in all types of infection with the variola-vaccinia virus, both in human and animal tissues They have been demonstrated in the epithelial cells of the skin, cornea, nose, mouth, larynx, pharynx, esophagus, sebaceous and meibomian glands, and conjunctiva, in the columnar cells of the nose, trachea, testes, and seminal vesicles, in the alveoli of the lung, in endothelial cells of vessels, connective tissue cells, and fibroblasts in infected tissue cultures and chorio-allantoic membranes

Intranuclear Inclusions

Intranuclear inclusion bodies occur most typically in variola, to a lesser extent in alastrim, but not in vaccinia, their characters have been described by Torres (1935–6) as follows

(*a*) Cytoplasmic and nuclear inclusions are not found in the same cell

(*b*) The cells with nuclear inclusions are found mainly at the bottom of the

vesicle, while those with cytoplasmic bodies are more numerous in the cells of the middle part of the malpighian layer.

(c) The intranuclear inclusions of variola may have various forms First, there may be an irregular mass (or small masses) of varying size which appears acidophilic; the nuclear membrane is little altered. Second, the inclusion may fill the whole nucleoplasm and be separated from the membrane by a clear space. Third, the inclusion may be formed by one or more regular ovoid or spherical acidophilic bodies.

Brinckerhoff and Tyzzer (1906) reported that the intranuclear forms occur only in small numbers in the primary skin lesions of inoculated monkeys, but in far greater numbers in the orang-utan. They reported also that nuclear forms are found only occasionally in the generalized lesions developing after dermal inoculation of the monkey with variola. They are, however, numerous in the eruptions which follow intravenous inoculation.

Comparisons between Inclusions of Alastrim and Variola

The South American workers, Torres and Teixeria, have paid particular attention to certain morphological differences between the inclusions of alastrim, variola, and vaccinia (Torres and Teixeria, 1933 *a–d*, 1934 *a, b*, 1935 *a, b*, 1937, Torres, 1935–6). Many of these observations have been carried out in monkeys.

(*a*) The inclusions of alastrim stain pale blue with safranine, but those of variola are red.

(*b*) The alastrim body is usually basophilic with HE, whereas the variola-vaccinia bodies are eosinophilic or polychromatophilic.

(*c*) In the later stages of infection, the alastrim body is usually single, or at most in pairs, whereas the other bodies are often multiple.

(*d*) The single inclusions of alastrim are larger than those of variola.

(*e*) The alastrim bodies never occupy an excavation in the nucleus, whereas this often occurs with variola.

(*f*) In the vesicle or pustule stage, the alastrim bodies are very scanty and best seen in the floor or walls, but in variola they are very numerous, and are seen best in the walls of the lesion.

(*g*) *Intranuclear inclusions of variola are acidophilic*, round or oval, and are separated by a halo from the membrane; they are single or multiple (up to 4) The inclusions of alastrim are retiform, and lack the definite structure of those of variola. Intranuclear inclusions do not occur in vaccinia.

(*h*) The nuclear membrane is considerably thickened in variola, whereas only moderately in alastrim The nucleus itself is diminished in variola, but increased in alastrim

Alleged Production of Guarnieri Bodies by Nonspecific Means

Certain authors claim to have produced Guarnieri-like structures by nonspecific means, e g, inoculation of croton oil, iodine, and other irritants (Ferroni and Massari, 1893), and inoculation of cultures of *B coli* or *B. paratyphosus* A (Pot, 1930). The specific origin of Guarnieri bodies is, however, unquestionable, and it has been shown that they cannot be produced by the inoculation of chemical irritants, diphtheria or tetanus toxins, or bacterial cultures (Rhodes and van Rooyen, 1937 *a*, Squarcia, 1938)

The Biological Nature of Guarnieri Bodies

1. The protozoal theory.

That Guarnieri bodies are forms of a protozoal parasite was widely believed at one time Guarnieri, for example, applied the term *Cytoryctes variolae* to the



FIG 29A Electron microscope photograph of elementary Paschen bodies of vaccinia, shadowcast with chromium, × 25,000

bodies, under the impression that they were protozoal and possessed of a nucleus Calkins (1904) described a complicated life cycle. Magrath and Brinckerhoff (1904 *a*) also postulated a protozoal life cycle (see also Brinckerhoff, 1904; Tyzzer, 1904)

2. The possible rôle of nuclei and nucleoli.

Certain authors suggested that Guarnieri bodies are composed of extruded nucleoli (Hammerschmidt, 1919, Ludford, 1927–8) It was also suggested that Guarnieri bodies may be nuclear fragments (Babès, 1894, Salmon, 1897). Eberbeck (1929) believed that Guarnieri bodies are derived from nuclei, nucleoli, and chromatin particles

That Guarnieri bodies contain chromatin appeared unlikely from the work of Cowdry (1922), who found that they do not give a positive Bensley-MacCallum test for iron However, more recently the nuclear theory has received support from Milovidov (1933–4) who has found that Guarnieri bodies give a positive nuclear reaction for thymonucleic acid (Feulgen test)

Others have also found that the Guarnieri body gives a positive Feulgen test, but do not thereby believe that the inclusion is necessarily composed of nuclear material (Haagen, 1937 *b*, Haagen and Kodama, 1937, Robinow and Bland, 1938, Bland and Robinow, 1939)

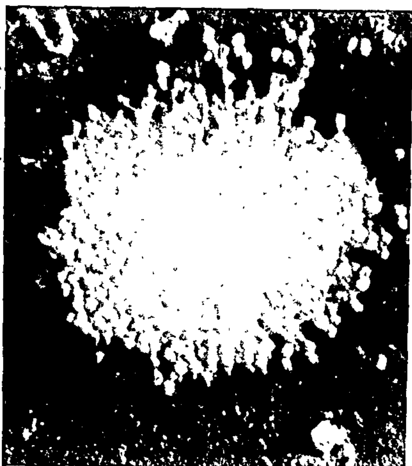


FIG 29B Electron microscope photograph prepared from smallpox vesicle fluid provided by *Dr E S Horgan of Khartoum*, showing Guarnieri inclusion body Shadowcast with chromium, × 8,000

3. The rôle of phagocyted red cells.

Woodcock (1921) suggested that Guarnieri bodies are phagocyted erythrocytes

4. Paschen's (1932) views.

Paschen holds that no single explanation can account for the Guarnieri body, and offers the following alternatives

(*a*) Guarnieri bodies are phagocyted leukocytes, sometimes perhaps a whole leukocyte, or a large part of it, is engulfed, at other times it is only the pyknotic nucleus

(*b*) Nucleolar extrusion occurs and gives rise to Guarnieri bodies

(*c*) Extrusion of chromatin takes place from the nucleus into the cytoplasm

(*d*) The cytoplasmic bodies are formed of a chromatin and a plastin component Probably vaccinia exerts a "toxic" effect which causes the normally united chromatin and plastin to dissociate and gives rise to inclusions (see also Nauck and Paschen, 1932 *a*)

(*e*) The small granular types of structure are probably true virus colonies

5. The rôle of other cellular constituents.

Cowdry (1922) found that Guarnieri bodies do not stain with Janus green for mitochondria, and that the result of silver preparations makes it unlikely that they

are constituted by the Golgi apparatus Ludford (1927–8), however, carried out a study of vaccinial inclusions in the skin of the chick and arrived at somewhat different conclusions He found that they arise as minute granules, which increase in size and become vesicular. The vesicles become covered with a strongly osmophilic substance He stated that the bodies are intimately associated with the Golgi apparatus in their early stages of development.

6. The Guarnieri body as a virus colony.

von Prowazek and his coworkers held that small elementary bodies enter the cell, where they increase in size to become initial bodies. These bodies measure up to 4 μ in diameter, and may divide up into elementary bodies again, or may become surrounded with a covering of reactionary material (chromatin and plastin) derived from the cell, thus constituting Guarnieri bodies (von Prowazek, 1905 *a*, *b*, 1910, 1912, von Prowazek and Aragão, 1908, 1909, von Prowazek and Miyaji, 1914, von Prowazek and Yamamoto, 1909). Hallenberger (1918) also supported these views He regarded the Guarnieri body as a defensive structure inclosing the virus particles, the virus initial body being covered with chromatin and plastin layers

For many years authors have realized that cells infected with vaccinia virus

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some time ago that the Guarnieri body is a product of the virus itself, that is to say, an intracellular colony (Ungermann and Zuelzer, 1920, Gins, 1922).

Now it will be recalled that the inclusions of mouse ectromelia and fowl-pox have been shown to be infective, and to contain a mass of elementary bodies The inclusions of molluscum contagiosum and psittacosis likewise contain countless elementary bodies Naturally one is led to inquire whether the Guarnieri body too consists of a colony of elementary bodies. Modern work with infected tissue cultures and chorio-allantoic membranes has shown quite definitely that such is the case, and the most important observations will now be cited in detail

(*a*) *Goodpasture* et al (1932), working with the infected chorio-allantoic membrane of the fertile hen's egg, found that inclusions developed in ectodermal cells before there was any evidence of cellular infiltration Almost all these cells in the recently infected areas showed inclusions, which were granular and amorphous, and sometimes occurred in irregular clumps When single inclusions were present, they were often triangular, with the base of the triangle toward the nucleus. Similar

(*b*) *Tang and Wei* (1937), by microscopical examination of chorio-allantoic lesions in the fresh state (with or without cresyl blue or neutral red), noted the occurrence of granular bodies in cells at the periphery of the pocks These structures were present in large numbers, were round, oval, or bacillary in shape, and occurred in a sort of matrix near the nucleus, some of the bodies showed brownian movement Smaller forms were also seen in the cytoplasm of squamous cells, fibroblasts, and even polymorphs It seemed that the larger forms appeared first and that after the development of necrosis the smaller forms were found However, even in the earliest stages some small forms could be found, and it appeared that there must be 2 phases of multiplication of the virus particles In the one type, there is a simple multiplication, in which the small forms divide, in the other type, multiplication occurs, but the particles become entangled in a matrix. The latter type is probably only found in the early stages

(*c*) *Hummelzwen* (1938) employed a most ingenious method of studying the

living chorio-allantoic membrane (duck's egg) *in situ*, using a Heine Ultropak[1] for annular oblique incident illumination. The vaccinial lesions consisted of a mass of infected cells with infiltrating leukocytes, and appeared heaped up and opalescent. At the edge, however, the tissue was not so opalescent, because the cells were not degenerate. Virus bodies could be first seen on the second day after inoculation, when there was a narrow peripheral zone of brilliant particles vibrating in constant brownian movement in a matrix of low viscosity. With the progress of infection, the virus particles increased in numbers, such an increase being always accompanied by a corresponding increase in the volume of the matrix. Sometimes the virus-containing part might become separated from the rest of the cell, the elementary bodies still being retained in the matrix, however. From these studies, Himmelweit concluded that the Guarnieri body is composed of a collection of elementary bodies contained in a matrix.

(*d*) Downie and Dumbell (1947 *b*) studied the inclusions of variola in the chorio-allantois. They could be found even after 24 hours; the inclusions were in the form of irregular eosinophilic granules. Single homogeneous bodies were not found The inclusions of variola were more coarsely granular than those of vaccinia.

Other authors who have demonstrated Guarnieri bodies in the infected chorio-allantoic membrane (of the hen's egg) include Goodpasture and Buddingh (1934), Gross (1937), Mesrobeanu (1937), and Buddingh (1938 *b*).

(*e*) With regard to Guarnieri bodies in *tissue culture*, these have been found to develop in corneal cells prepared by the tube technique, both in epithelium (Rivers, Haagen, and Muckenfuss, 1929 *a*, *b*, *c*), and in fibroblasts (Rhodes and van Rooyen, 1937 *b*); Mitamura *et al.* (1933) and Feller, Enders, and Weller (1940) have also found Guarnieri bodies. Bland and Robinow (1939) showed that when infected corneal epithelial cells were grown in tissue culture, vaccinia virus underwent a progressive and obligatory process of intracellular development. Elementary bodies penetrated the cytoplasm, became enlarged to form inclusions, and altered

homogeneous bodies, then, large homogeneous bodies, then, small networks, later, medium networks, and finally, large networks During the greater part of this process, elementary bodies were absent from the cells, presumably being within the inclusions. At the end of the process, elementary bodies were again found in increased numbers in only those cells containing the largest type of inclusion The inclusions are believed to be an obligatory stage in the multiplication of the virus, and to consist of colonies of elementary bodies enveloped in a matrix, thus supporting von Prowazek's original claim that Guarnieri bodies are *Chlamydozoa*

More recently, Merling has carried out direct microscopic observations on infected cells He produced vaccinial keratitis in the rabbit, detached the corneal cells, and placed them in saline or Tyrode in sealed slides (1940 *a*) Virus was found in the cells 24 hours after inoculation The surface of the cell was first studded with EB's, which later sunk in The cytoplasm appeared dissolved, and the collection of EB's was in brownian movement One cell might contain several of these Guarnieri type inclusions, the average inclusion containing about 150 EB's Disks were also observed in the cytoplasm of the cell, or the inclusion, spheres were found in the inclusions and lying outside the cell EB's were seen to develop into disks, at the periphery of which nodes appeared These formed into separate EB's, and were released into the liquefying center of the disk, giving a small inclusion The inclusions measured 0.5 to 15 μ, the spheres 0.5–1.0 μ, EB's 80–120 mμ, and disks 0.15–0.2 μ.

[1] Ernst Leitz Wetzlar

In other experiments (1940 *b*), observation was carried out for a longer period, and certain additional changes described The commonest long-term change was the appearance of cell extrusions originating from Guarnieri bodies, and displaying a definite increase in the number of virus bodies. Rarer forms were cyst-like extrusions filled to capacity with all forms of virus. He concluded that virus continues to live after the death of the host cell, and confirmed these observations in later work (1943). Virus was also found to grow inside leukocytes, and formed colonies (1945).

(*f*) It is possible that valuable observations may be made by electron microscopy of tissue or exudates (see Fig 29B).

CHAPTER XXX

THE VARIOLA-VACCINIA VIRUS CULTIVATION

INFECTION OF THE EGG WITH VARIOLA

MATERIAL from smallpox lesions produces typical pocks on the chorio-allantois, and this has been made use of in diagnosis (see Ch. XXVII, Lazarus *et al*, 1937,[1] Buddingh, 1938 *b*, 1943, Nelson, 1939, 1940 *b*, 1941. 1943, Irons *et al*., 1941, Bohls and Irons, 1942, North, 1944, North, Broben, and Mengoni, 1944, Downie, 1946, Downie and Dumbell, 1947 *a*, *b*). EB's and inclusions can be found readily. Variola passed on the chorio-allantois does not apparently become converted into vaccinia Buddingh (1938 *b*) found that the pocks were produced by ectodermal hyperplasia, with central necrosis Vesicle formation was observed in the hyperplastic epithelium Small Guarnieri bodies were seen in the less degenerate cells The mesoderm showed edema and infiltration with mononuclears and polynuclears. Entodermal cells were also hyperplastic. Downie and Dumbell recommend examination of the membranes after 48–72 hours. The number of pocks may be considerable, as they are small and dome-shaped. They found inclusions within 24 hours of infection in the form of fine acidophilic granules in the ectoderm Large homogeneous bodies, as found in the cornea, were not found. The chick embryo was not significantly infected. Downie and Dumbell compared the lesions induced by variola and vaccinia, vaccinial lesions were larger, flatter, more necrotic, showed hemorrhage more frequently, and histologically there was more destruction and less proliferation

INFECTION OF THE EGG WITH VACCINIA

Following the pioneer work of Goodpasture, Woodruff, and Buddingh (1932), who showed that vaccinia virus could proliferate when inoculated onto the chorio-allantois of the fertile egg, numerous other workers have studied this reaction[2] (Lehmann, 1934, Pandit and Rao, 1935, Shah, 1935, Sporzynski, 1935, Torres and Teixeria, 1935 *c*, Molina, 1937 *a*, *b*, Stevenson and Butler, 1939, and others, see below). In addition, many workers have grown virus in eggs for use in vaccination (see p 382). The virus infects most regularly by the chorio-allantoic route Hen's eggs are invariably used, the turtle egg is also susceptible (Harris, 1945)

Pathology of Infected Membranes

Naked-eye, the infected membrane usually shows the development of whitish, raised, pock-like lesions known as plaques, which may become confluent

1 *Goodpasture*, W[illegible] membranes in their or[illegible] cine, they found that [illegible] opaque about the site [illegible] this was thick, gray, opaque, and flecked with small hemorrhages Histologically, the membrane was considerably thickened, due to inflammation and hemorrhage At the edge of the lesions there was edema, some hemorrhage, and slight hyperplasia of the ectoderm and endoderm Guarnieri bodies were seen in ectoderm In older areas the ectoderm was necrotic, there was an exudate in the mesoderm of red cells, white cells, and large mononuclears, although the endoderm was hyperplastic, no necrosis was evident They concluded that the virus affected primarily the ectoderm, the mesoderm less, and the endoderm least of all

2. *Tang and Wei* (1937) examined the lesions in infected membranes after 48

1 References are appended at the conclusion of Ch XXXV, p 403 et seq

2 General details of egg technique are discussed in Ch XIII

hours, and found the following changes (the strain of virus used had originated in dermal lymph): Accumulations of cells were seen, due to ectodermal proliferation and cellular infiltration, and in some of these areas necrosis had given rise to umbilication Where there was no necrosis, there was a translucent superficial opacity which faded into the normal tissue at the edges of the lesion Cells at the periphery of the lesion were very irregular in size, and contained numbers of granular structures

3. *Levaditi and Voet* (1937) studied the reaction to both dermo- and neuroviruses The first change noted was an ectodermal thickening, from the 18th hour onward The epithelial cells showed numerous mitotic figures, the cytoplasm was swollen, vacuolated, and contained inclusions A mesodermal reaction was noticed from 25 hours onward, being evidenced by infiltration with leukocytes and histiocytes, there was a hyperplasia of the vascular endothelium and a new formation of vessels. At this time, vesicle formation was noted in the epithelial cells, and there were more Guarnieri bodies Between the third and fourth day ectodermal proliferation was very marked, and cell nests were formed in the mesoderm. Later, pustules were produced

4. *Stevenson and Butler* (1939) reported that to naked-eye observation there was no difference detected in the course of over 100 egg passages of dermal virus After 4 days' growth the most characteristic lesion was a confluent plaque, without hemorrhage.

5. *Buddingh* (1936 *a*) has compared the pathological features of membranes infected with neurotesticular and dermal strains of virus The dermal strain showed a special tendency to involve ectoderm, while the neurotesticular was characterized by hemorrhage and a predilection for mesoderm.

6 *Keogh* (1936) studied the difference in the reactions produced by dermal and neurotropic viruses The pocks produced by the neurovirus strain were smaller and more defined than those produced by dermal virus, but there was more necrosis with the former.

7 *Mesrobeanu* (1937) also studied the histological changes in infected egg membranes, according to whether dermo- or neuro-vaccinia was used. With dermovaccine, the changes were more pronounced in the first 48 hours and were more widespread The lesions produced by neurovaccine were more focal, there were fewer leukocytes in the mesoderm, in fact there was a special tendency to histiocytic increase

Normal Corpuscles

In their work on the infectivity rate of EB's prepared from chorio-allantoic suspensions, Levaditi *et al* found that certain "normal corpuscles" were present that closely resembled vaccinial EB's (Levaditi, 1939 *c*; Lépine, Levaditi, and Giuntini, 1942 *a*, *b*, Pérault, 1942) These normal corpuscles were particularly evident in suspensions prepared from membranes shortly after infection, after 2–4 days' incubation the vaccinial EB's were more numerous than the normal corpuscles

The Effect of Passage on the Properties of Egg Virus

There is some difference of opinion as to whether egg passage alters the properties of virus. Lehmann (1934), for example, found that passage increased the virulence of virus for the rabbit. Tang and Wei (1937) found that virulence increased *pari passu* with the number of transfers on the chorio-allantois After a number of passages, rabbits injected with suspensions of infected membrane often died Further, the chick embryos were frequently killed in the later passages, although this had not occurred at the beginning of the work, from the 15th generation onward, pustules occurred on the toes or beak of the embryo

On the other hand, Stevenson and Butler (1935) detected no differences between 12-passage virus and calf lymph on skin inoculation of monkeys and calves

Continuing their work, they reported (1939) that somewhere between the 24th and 55th subcultures, and in later passages, a 1/100 dilution of chick membrane lymph gave a somewhat similar infective response to that produced by a 1/1,000 dilution of calf lymph. Echenique-Guzman (1937) found that repeated egg passage did not augment the infectivity of a dermovirus or of a neurovirus strain. Kunert (1935) actually found that the effect of passage on the Berlin strain of virus was to induce a decrease in its virulence. Rabbit passage, however, restored its original activity.

Buddingh (1936 *a*) studied the effect of passage on the properties of a neurotesticular strain of virus which produced a hemorrhagic lesion on the chorio-allantois and the rabbit's skin, and found that the hemorrhagic nature of the lesions was maintained through 50 egg transfers.

Stevenson and Butler (1939) have carried out considerably over 100 egg passages with a dermal strain of virus. With regard to the lethal effect on the embryo, they found that this was slight in the first 2 passages or so, from the 3rd to the 14th (in some cases the 29th) embryos, however, died more frequently; finally, from the 50th to the 126th the virus was not lethal to the embryo.

Further, they reported that the reactions produced by inoculation of the skin of rabbits, monkeys, and calves with egg virus resembled in every important respect those produced by calf lymph. The lesions were, however, slightly slower in development, the surrounding skin was less congested, and the crusts were slighter. They failed to find that growth in the egg endued the virus with any tendency to generalize on inoculation, nor did any neurotropic tendencies present themselves on direct intracerebral injection.

Buddingh (1943) examined a strain after 240 passes. The lesions produced in the rabbit skin were milder than those produced by calf lymph

The Chick Embryo in Vaccinial Infection of the Egg

Buddingh (1936 *b*) has published a detailed study of the reaction of the chick embryo to infection of the chorio-allantois with dermal virus. In short, he found areas of focal necrosis in the liver and spleen, with pocks on the skin and mucous membranes. Virus was widely distributed—occurring in the liver, spleen, skin, intestines, kidney, heart, marrow, but only to a slight extent in the brain. Stevenson and Butler (1939) did not, however, obtain nearly such severe reactions in chick embryos.

The embryo may fail to grow at the normal rate (Hoffstadt and Tripi, 1946 *b*).

Applications of the Egg Technique

1 *For human vaccination* This is probably the most important application of the egg technique. Infected membranes may be ground up and used as a source of vaccine for human inoculation This question is discussed more fully in the section on vaccination (see p 382).

2. *Titration of virus and antiviral sera* Keogh (1936) has shown that inoculation of the chorio-allantois can be used as a means of estimating the potency of vaccinial suspensions. He has found that if suspensions of virus are inoculated into eggs in suitable dilutions, then discrete lesions are produced, and that the titer obtained by counting these pocks corresponds to that yielded by intradermal inoculation of animals. He has also shown that immune sera may be titrated by mixing with virus and inoculating eggs Account is then taken of the percentage reduction effected in the number of pocks developing on the chorio-allantois. Burnet and Faris (1942) also studied the titration of virus, but Haagen and Crodel (1939–40) did not agree that the chorio-allantois could be used as an accurate method of titration.

3. *Other applications.* Keogh (1937) studied the kinetics of the disinfecting action of formalin on vaccinia virus by the chorio-allantoic technique. Without

entering into details, it appeared that the formula $Kct = \log \frac{V}{v}$ applied to this reaction (V = original number of virus particles, v = number of particles surviving the disinfection); further the reaction was approximately doubled by raising the temperature from 23° to 37° C. The membranes can be used as a test of inactivation of virus by chemicals (Dunham and MacNeal, 1942; Cutting *et al*, 1947).

The infected chorio-allantoic membrane has been used as an antigen in the complement fixation test (Kunert and Wenckebach, 1936).

Other Routes of Inoculation

It has been reported that the egg is susceptible to yolk sac inoculation, and less readily susceptible to infection in the allantoic sac (Groupé and Rake, 1947).

IN VITRO CULTIVATION OF VACCINIA

1. Media Containing Fungi or Yeasts

It has been claimed that growth of vaccinia can be obtained in media containing fungi (Isabolinski *et al.*, 1935), or yeast (Silber and Wostrouchowa, 1933 *a*, *b*, Khurgina, 1935, Silber, 1935) Amies (1934 *b*), Voet (1935), and Lenz (1937) were unable to obtain growths.

2. Growth in Cell-free Media

Claims have been made that vaccinia [illegible] free of whole or viable cells, known a [illegible] 1930, 1931; McClean and Eagles, 1931, E [illegible] also Ch XII). Although virus may survive in the presence of [illegible] substances elaborated by cells (Muckenfuss and Rivers, 1930, Muckenfuss, 1931), Eagles's experiments have not been generally confirmed (see Krontowski *et al*, 1933, Rivers and Ward, 1933 *a*, *b*)

3. Growth in the Presence of Living Cells (Tissue Cultivation) [1]

Certain preliminary observations showed that some survival, and even definite proliferation, of vaccinia virus occurred in association with animal tissue suspended in serum or other nutrient fluid (Steinhardt *et al*, 1913, Steinhardt and Lambert, 1914, Harde, 1916; Fornet, 1922; Parker, 1923–4, Parker and Nye, 1925, Craciun and Oppenheimer, 1926, Carrel and Rivers, 1927, Levaditi, 1927, Haagen, 1928).

[illegible]

land, Laing, and Lyth, 1932) In later work, Maitland and Laing (1941) showed [illegible]

[illegible]

cinia using minced chick embryo as a source of living cells (see also Rivers and Ward, 1931).

At the present day, the tissues most commonly used to obtain growths of vaccinia are rabbit testis or kidney, or minced chick embryo, spleen and cornea may also be employed, but are not so effective, cultures of Kupffer cells (Beard and Rous, 1938), and rabbit mononuclear cells have also been used (Florman and Enders, 1942).

The results of the pioneer workers have been abundantly confirmed, and many authors have succeeded in cultivating vaccinia virus in the types of tissue culture

[1] For fuller details of tissue cultivation technique see Ch XII

introduced by Maitland and by Rivers (Eagles and McClean, 1929, 1930, Hauduroy, 1929, Nye and Parker, 1929, Rivers, Haagen, and Muckenfuss, 1929 *a*, Kimura and Fujisawa, 1929–30; McClean and Eagles, 1931, Breinl, 1933; Haagen, 1933, Rivers, Sprunt, and Berry, 1933, Herzberg, 1935, Rao *et al.*, 1935–6, Haagen, 1936 *b*, Haagen and Crodel, 1936–7, Galli, 1937, Plotz, 1937, 1938 *a*, *b*, Plotz and Lépine, 1938, Enders and Florman, 1942, Thompson and Coates, 1942 and others mentioned below.

Kurotchkin (1939) has claimed that growth occurs in chick embryo tissue on the surface of agar slopes Thompson and Coates (1939) have grown virus in columns of Maitland's medium up to 115 mm deep Yaoi and Arakawa (1939 *a*) grew vaccinia in deep columns of Tyrode and minced chick embryo, with oxygenation The virus grows well in roller tube cultures, rapidly increases to maximum titer, and remains at or near maximum for at least 9 weeks, living cells are essential, and it seems that virus is being continually produced, not merely preserved (Feller, Enders, and Weller, 1940).

The Effect of Passage on Culture Virus

Certain strains of virus may be readily adapted to growth in tissue culture, and numerous transfers made. In other cases, however, continued propagation may prove rather difficult. When culture virus shows evidence of becoming reduced in potency, one or more passages through rabbits serve to restore its original characteristics (Ch'en, 1933–4, Coffey, 1934). Although culture virus does not usually alter in any important respect with regard to the nature of the lesions produced on injection of animals, Haagen *et al* (1932) reported that a strain altered to produce hemorrhagic and necrotic lesions in the rabbit's skin. Parker, Bronson, and Green (1941) worked with a strain that had lost most of its virulence for rabbits on prolonged cultivation, it did not prove possible to restore this virulence by various procedures.

Preservation of Culture Virus

Various methods have been adopted to store tissue culture virus in a suitable virulent condition For example, it can be stored at +5° C. for at least 64 days, if mixed with glycerol and sealed (Li and Rivers, 1930) Later it was reported that virus could be stored for at least a year in 50 per cent glycerol at −10° C, and at +3° C. (Rivers and Ward, 1933 *c*) Mixed with 2½ per cent. gum acacia, frozen, desiccated, and sealed *in vacuo*, virus remains active for a month at 37° C (Rivers and Ward, 1935)

If the culture fluid is diluted with equal parts of horse serum and desiccated *in vacuo* from the frozen state, virulence can be maintained at 4° C for at least 69 days (Lloyd and Mahaffy, 1935–6) Togounova *et al* (1935) have claimed that on ice, or desiccated, culture virus survives for over one year

Applications of the Tissue Culture Technique

1 Tissue culture may be used as a source of vaccine for human inoculation (see Ch. XII)

2 Tissue culture has been employed as a method of studying the theoretical aspects of the vaccinial antigen-antibody reaction (Stoel, 1930–1, Sabin, 1935 *b*, Magrassi and Hallauer, 1936)

3. Culture virus may be used as an antigen for immunizing animals. For example, Maitland and Laing (1930) found that the serum of rabbits immunized against culture virus fixed complement with calf lymph Togounova (1935) found that the sera of animals immunized with culture virus developed virucidal, agglutinating, and complement fixing antibodies (see also Plotz, 1938 *d*)

4. Culture virus may be used as an antigen in serological tests For instance, Maitland and Laing (1930) found that antivaccinial serum fixed complement in the presence of culture virus (see also, Gilmore, 1931, Barg and Rudenko, 1934)

CHAPTER XXXI

VARIOLA-VACCINIA VIRUS· HEMAGGLUTINATION

It has been found that variola and vaccinia virus may agglutinate chick red cells. A similar type of agglutination, which differs somewhat from that produced by influenza (Ch LXI), is given by the virus of mouse ectromelia (Nagler, 1942,[1] North, 1944, Burnet, 1945, 1946, Shubladze and Soloviev, 1945, and others to be mentioned). The hemagglutination reaction with chick cells may be referred to as the CCA reaction, and the vaccinia and ectromelia hemagglutinins as V and E HA

SOURCE OF V AND E HA

Hemagglutinin is most readily demonstrated in suspensions of infected chorio-allantoic membranes

Suspensions of organs of chick embryos inoculated intravenously with ectro-

1. VHA was found in the early stages of dermal infection in rabbits, and the ratio between hemagglutinin (HA) and virus (V) titers could be expressed in the form HA/V 10^{5}. The ratios in the superficial scrapings from several rabbits 3 days after inoculation varied from 0 08 to 1 6, whereas in the skin suspensions they were much higher, 4 4 to 23 That is to say, the ratio of VHA to virus was higher in the deeper layers than in the superficial scrapings

2. Virus was found in almost undiminished amount in the scab formed after 7–8 days, and in the underlying skin, but at this time there was a rapid disappearance of hemagglutinin from both sites From 7 days onward, antihemagglutinins were found in the skin

3 Crude calf lymph was emulsified in saline to form a 20 per cent. suspension, and was then centrifuged The supernatant fluid had no hemagglutinating activity. It was then titrated for antihemagglutinin against 5 hemagglutinating doses of VHA, and a titer of 25–35 was obtained. The same fluid was tested against ectromelia and influenza A hemagglutinins with the following results, vaccinia 28, ectromelia 8.5, influenza less than 4 This behavior, and the results of further tests, suggested that the inhibitory agent in calf lymph was an antibody resembling serum antibody.

4 In chorio-allantoic lesions due to vaccinia or ectromelia there was also a difference in the relative virus hemagglutinin content of the superficial (necrotic) and deeper tissues The HA/V 10^{-5} ratios for the deeper portion of the membrane lesions were 10–20 times as high as those for the superficial scrapings If saline suspensions from superficial chorio-allantoic lesions were allowed to stand in the refrigerator for 10 or more days, their hemagglutinating activity showed a marked increase This effect was probably due to the liberation of lipoid material, and was inhibited by dilute normal serum These examples stress the importance of controlling the specificity of hemagglutination reactions with separate tests employing immune and normal serum

The points of interest brought out in this work were the low hemagglutinin titer of the superficial layers of rabbit skin and chorio-allantois as compared with their high virus content The hemagglutinin is presumably produced predominantly

[1] References are appended at the conclusion of Ch XXXV, p. 403 et seq

introduced by Maitland and by Rivers (Eagles and McClean, 1929, 1930, Hauduroy, 1929, Nye and Parker, 1929, Rivers, Haagen, and Muckenfuss, 1929 *a*; Kimura and Fujisawa, 1929–30; McClean and Eagles, 1931; Breinl, 1933; Haagen, 1933, Rivers, Sprunt, and Berry, 1933, Herzberg, 1935; Rao *et al*, 1935–6; Haagen, 1936 *b*, Haagen and Crodel, 1936–7, Galli, 1937, Plotz, 1937, 1938 *a*, *b*; Plotz and Lépine, 1938, Enders and Florman, 1942; Thompson and Coates, 1942 and others mentioned below.

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AGGLUTINATION BY LIPOIDS

Burnet and Stone (1946) showed that various tissue lipoids, e g, from the normal chorio-allantois, give hemagglutination resembling that produced by VHA. This type of hemagglutination, however, is readily inhibited by dilute normal serum, whereas virus hemagglutination is not Stone has carried out more extensive experiments

1. VHA and EHA are inactivated by phospholipoid-splitting enzymes, such as *Cl welchu* α toxin, and cobra venom (Stone, 1946 *a*). When VHA was incubated at 37° C with toxin it was progressively reduced in titer. No decrease in titer followed treatment with heated toxin for 4 hours, although there was slight inactivation after 24 hours The inactivation of VHA by toxin was inhibited by the inclusion of antiserum to *Cl. welchii* α toxin type A. There was no apparent destruction of VHA in the absence of calcium ions. As calcium or magnesium ions are required for the action of the α toxin (lecithinase) of *Cl welchu*, it is suggested that lecithinase is responsible for the inactivating effect of toxin. A preparation of hyaluronidase, also produced by certain strains of *Cl. welchu*, did not inactivate VHA.

EHA was similarly susceptible to inactivation by *Cl welchii* type A toxin, the capacity to agglutinate mouse cells appeared to be destroyed in parallel with hemagglutinating capacity for fowl cells.

Cobra venom, which contains a different type of lecithinase rapidly destroyed both VHA and EHA

It seems highly probable that lecithinase is responsible for the inactivation of VHA and EHA by *Cl welchu* toxin It is concluded that VHA and EHA contain a phospholipoid, most probably lecithin, and that enzymic breakdown of this part of the complex destroys its hemagglutinating properties.

2 Attempts to isolate the active hemagglutinating principle from extracts of chick embryo liver, and baker's yeast, yielded little information (Stone, 1946 *b*) From a limited survey of the hemagglutinating properties of various lipoids, it did not appear that [illegible]
in tissue extract
cluding beef hea
that a mixture c
and the lecithin could be replaced by sphingomyelin, and the cholesterol by (*a*) ergosterol, maize sterols, or (*b*) cardiolipin without loss of activity. Taking a lecithin-cholesterol mixture as a typical example, the general properties were found to agree with those of the tissue lipoid hemagglutinin. In the bulk of the tests, the increased activity in mixed preparations of lipoids was demonstrable only with susceptible fowl cells. The weak hemagglutination of insusceptible cells which is shown by some compounds such as cholesterol and cardiolipin is actually depressed by the addition of lecithin

Stone concludes that the fact that alcoholic mixtures of purified lipoids (such as lecithin and cholesterol) are active hemagglutinating agents when diluted in saline, offers a reasonable explanation of the activity of tissue extracts In any such extract there will normally be a mixture of phospholipoids and sterols, and the main hemagglutinating activity can be accounted for by lecithin-cholesterol in animal extracts, and lecithin-ergosterol in yeast extracts It may be, as suggested by Eagle in connection with the Wassermann antigen, that the phospholipoid is distributed on the surface of cholesterol aggregates and stabilizes the cholesterol suspension in the aqueous medium Whether the two components of the mixtures have specific configurative parts to play in the hemagglutination reaction, or whether one merely serves as an inactive nucleus for the suitable distribution of the other cannot yet be determined

The depressant effect of lecithin on the agglutination of insusceptible cells by

in the epithelial cells, and it is suggested that it may be oxidized and destroyed in the superficial layers.

The failure of calf lymph of high virus content to show any specific hemagglutinin may be due partly to oxidation in the superficial layers, but there is in addition an actively inhibitory substance present, which is almost certainly antibody. This antibody is possibly produced in the immediate vicinity of the lesion.

The fact that nonspecific hemagglutinins, probably lipoid in character, develop in chorio-allantoic scrapings when left in the refrigerator, indicates an important potential source of error in experimental work.

CELLS AGGLUTINATED

Clark and Nagler (1943) found that about 50 per cent. of fowls give red cells agglutinable by vaccinia, and agglutinability develops in 2–4 weeks after hatching Variola agglutinates fowl cells less readily than does vaccinia

Fowls could be classified into 3 groups according to whether cells were agglutinated by both viruses, strongly, weakly, or hardly at all (Burnet and Boake, 1946).

Ectromelia agglutinates mouse cells, but vaccinia does not (Burnet and Boake, 1946). Pigeon cells have also been used (Burnet and Stone, 1946). Clark and Nagler (1943) tested a wide range of cells (see Ch. LXI).

CCA-INHIBITION

CCA is inhibited by antisera prepared experimentally in animals or obtained from convalescents. These antibodies develop after primary "takes" on vaccination or revaccination, and less markedly after accelerated or "immune" reactions on revaccination (Nagler, 1942, 1944).

Antiserum inhibits the agglutination caused by either variola or vaccinia (North, 1944) Inhibition can also be shown with ectromelia antisera (Burnet and Stone, 1946).

SOME CHARACTERS OF HEMAGGLUTINATION

No significant differences have been noted in the character of the hemagglutination produced by vaccinia and ectromelia viruses, but both ectromelia and vaccinia hemagglutinins differ considerably from the influenza-Newcastle type of agglutinins

1 Agglutination with vaccinia and ectromelia occurs best at 37° C, but that with influenza is best at lower temperatures. The apparent titer rises if cells and virus are allowed some time for interaction before being finally shaken and allowed to settle, especially when lower concentrations (1 per cent) of susceptible fowl cells are used (Burnet and Boake, 1946)

2 No evidence of elution of the hemagglutinins after prolonged contact has been obtained, susceptible fowl cells rendered inagglutinable to most influenza viruses are s [illegible] ia hemagglutinin (Burnet and Boake, [illegible] if VHA is removed by exposure to [illegible]

3. The hemagglutinins of vaccinia and ectromelia viruses are soluble and distinct from the virus particles, as can be shown by suitable absorption experiments The hemagglutinin of influenza virus is a function of the elementary bodies That VHA and EHA are distinct from the virus particles has been demonstrated by Burnet and Stone (1946) The hemagglutinin is readily removed from stock virus suspensions by absorption with susceptible fowl red cells, to a very limited extent with insusceptible cells and not at all by embryonic cells The virus content is essentially unaltered by these procedures. Centrifugation of infective suspensions at 15,000 r p m results in practically all the virus being removed from the supernatant fluid, which fluid however shows no significant loss in hemagglutinins

CHAPTER XXXII

THE VARIOLA-VACCINIA VIRUS: EFFECT OF PHYSICAL AND CHEMICAL AGENTS[1]

THE EFFECT OF PHYSICAL AGENTS ON VACCINIA VIRUS

Heat The virus is fairly readily destroyed by heat, for example, when heated in Locke's solution to 55° C. for 20 minutes, or to 37 5° C for 24 hours (Nye and Parker, 1929)[2] It has, however, been claimed that although neurovirus is destroyed at 55° C for 1 hour, dermovirus is not (Levaditi and Nicolau, 1923 *a*) With regard to the time of survival at 37° C, it has been stated that suspended in a special buffer solution, virus retains its infectivity for as long as 66 days (Silber and Wostrouchowa, 1932). The effect of heat on antigenic properties is discussed on p. 359

Cold. As in the case of other viruses, infected suspensions can be stored in the cold for prolonged periods Buddingh (1938 *b*), for example, found virus (suspended in saline) to retain its activity at 0° C for at least 5 weeks For conservation of infected egg membrane, Haagen (1939) has recommended that it be sealed hermetically and kept at 4° C.

Desiccation Vaccine virus can be maintained in an active condition for prolonged periods if infected tissue, such as testis, is rapidly desiccated *in vacuo* (preferably from the frozen state), and stored in sealed test tubes, it may survive for 3 years (Hoffstadt and Tripi, 1946 *a*) Dearing (1934) also carried out experiments on dried virus

Supersonic waves Although crude suspensions were not found to be inactivated by supersonic waves, chemically "purified" suspensions were destroyed by very short exposures (Yaoi and Nakahara, 1934).

Ultrapressure. Vaccinia has been found to resist 1,800 atmospheres for 45 minutes, but to be destroyed by 4,500 atmospheres applied for the same time (Basset *et al*, 1933).

pH Moriyama (1937 *a*) found that activity was slowly lost when hydrochloric acid was added to vaccinia virus to produce a pH of between 3 and 5. The addition of caustic soda at once restored activity, but this reaction eventually became irreversible

The electrical charge of vaccinia virus A number of authors has studied the

(Rivers and Gates, 1928, Kirstein, 1929, Linden and Schwarz, 1931, Barkad *et al*, 1932), and to x-rays (Lacassagne and Nyka, 1938) Polonium destroys the virus (Bonet-Maury and Olivier, 1938) *In vivo* it has been shown that ultraviolet radiations may prevent the development of an eruption due to the inoculation of virus (Carnot *et al*, 1926) Rivers *et al* (1928) found that rabbit's skin treated for a few minutes with ultraviolet light was less susceptible to vaccination than was untreated skin Herzberg (1933 *b*) found that ultraviolet irradiation applied up to the 8th hour after inoculation prevented the appearance of pustules Alpha rays of radon destroy infectivity (Levaditi and Pérault, 1943)

Le Fèvre de Arric (1927) reported that the exposure of the rabbit's skin to x-rays rendered it insusceptible to vaccination

[1] See also the effect of physical and chemical agents on EB's (p 338) The reported effects of these agents on EB's, and less purified forms of virus, are not necessarily identical

[2] References are appended at the conclusion of Ch XXXV, p 403 et seq

cholesterol and cardiolipin would explain the complete inactivity of tissue extracts against insusceptible cells, even though they contain active compounds such as cholesterol and cardiolipin

The properties of hemagglutination and activity in WR both appear to be based on suitably dispersed lipoid combinations, but the Wassermann activity is much more restricted than the agglutinating effect, which is shown by mixtures serologically inert.

Lipoid hemagglutination is of biological interest, chiefly in its bearing on the problem of the nature of the specific hemagglutinins of vaccinia and ectromelia viruses. The provisional picture is of the vaccinia hemagglutinin as a relatively well-defined and stable complex of a virus antigen and a phospholipoid, the latter being responsible for union with the surface of the cell

THE PROTECTIVE EFFECT OF CERTAIN AGENTS ON THE ACTIVITY OF VACCINIA VIRUS

The activity of vaccinia virus can be maintained for considerable periods when suspended in the presence of certain substances e.g, broth, glycol, and glycerol, gum acacia (see Goodpasture and Buddingh, 1936); milk, lecithin, or egg yolk (Hirano, 1937), normal inactivated rabbit serum (Goodpasture and Buddingh, 1936); saccharose (Akasawa, 1936), cysteine hydrochloride (Long and Olitsky, 1929–30). Extensive experiments in this connection have been carried out by Buddingh (1938 *a*), using the following substances 30 per cent. gum acacia in saline; 3 per cent granular gastric mucin, equal parts sterile egg yolk and 50 per cent. glycerol, sterile 0 85 per cent. saline, normal inactivated sterile rabbit serum, normal inactivated sterile beef serum, and 50 per cent. glycerol in saline He found that egg-cultured virus retained some infectivity at 37° C. for at least 7 weeks when suspended in sterile rabbit or ox serum.

PHYSICAL AND CHEMICAL "PURIFICATION" OF VACCINIA VIRUS

Numerous attempts have been made to reduce the quantity of the noninfective material in vaccinial suspensions by elution, adsorption, precipitation, and other procedures (Awad, 1931, Sato and Kodama, 1931; Tang, 1932, Kodama, 1933 *a*, *b*, Yaoi and Kasi, 1929 *b*, 1931 *a*, 1933, Yaoi, 1931, 1934, 1935 *a*, *b*, 1936 *c*). The methods used have included the following adsorption with charcoal, glass, kieselguhr, and kaolin (Gins, 1914, Awad, 1931; Tang, 1932), adsorption and precipitation by hydroxides or phosphates of aluminium, iron, or zinc have also been used (Kodama, 1933 *a*, *b*). Kligler (1934–5) reported that by adsorption with kaolin, and successive elutions with ammonia, it was possible to obtain a potent suspension of virus, this

phenol and sodium chloride are finally added

The method of iso-electric precipitation of virus-containing suspensions was employed by Behrens and Nielsen (1935), who obtained water-clear virus suspensions with a high infective titer, from which up to 85 per cent. of protein was removed (see also Behrens and Morgan, 1932).

CHEMOTHERAPY OF VACCINIAL INFECTIONS

In general, vaccinia virus is little affected by any chemotherapeutic agent. For example, Andrewes, King, and van den Ende (1943) tested 74 compounds *in vitro*, but none was of any value

Sulfonamides have no effect on vaccinial infections (Bruni and Buda, 1939, Kolmer and Brown, 1941, Osberghaus, 1942, Thompson, 1947).

Thompson (1947), however, who studied a number of metabolites, metabolite-antagonists, and enzyme-inhibitors in infected cultures, found the following to be inhibitory dinitrophenol, malonic acid, cyanide, atabrine, proflavine, iodoacetic acid, and ascorbic acid

Antibiotics. Most workers have found that *penicillin* has no virucidal effect on the virus *in vitro* or *in vivo* in man, animals, cultures, or eggs (Andrewes, King, and van den Ende, 1943, Parker and Diefendorf, 1944, Diaz Romero, 1945, Kolmer

Filtration. Many workers have been unable to secure infective filtrates of vaccinia-infected tissue suspensions. Careful attention to a number of technical details is necessary, however, and if these are attended to, filtrability can be demonstrated (see also Ch. IV). Ward (1929), for instance, recommended (*a*) the use of fresh vaccine pulp; (*b*) the grinding of this pulp with pulverized glass (Pyrex brand) in a glass mortar, (*c*) the emulsification of pulp in hormone broth, (*d*) centrifugation, and the passage of the supernatant only through the filter. The following filters have all been shown to "pass" the infective agent of variola-vaccinia (the filtrate is, however, always considerably less infective than the unfiltered material): Berkefeld V, N, and W, Chamberland L_3; collodion membranes; Kitasato, Mandler (Negri, 1905, 1906; Carini, 1906; Nicolle and Adil-Bey, 1906, Casagrandi, 1908; Ward, 1929; Ward and Tang, 1929; Yaoi and Kasai, 1929 *c*, *d*; Monteiro and Godinho, 1930; Green and Eagles, 1931, Hirano, 1931 *a*, and others, see Thomson, 1924).

Centrifugation. Vaccinia virus is partly deposited by comparatively low-speed centrifugation, e g, at 6,000 r p.m. for 2 hours (Bland, 1929; see also Tang, 1930) Using an angle instrument, EB's can be deposited more quickly and at a lower speed.

PHOTODYNAMIC INACTIVATION OF VACCINIA VIRUS

A number of dyes have been found to destroy vaccinia virus *in vitro* in the presence of light. Friedberger and Yamamoto (1909 *a*, *b*), for example, found that neutral red (1/10 million) destroyed virus in sunlight in 7 hours. Eosin and rose bengal solutions have a similar photodynamic effect (Barkad *et al.*, 1932).

Herzberg (1931 *a*, *b*) has also studied this question and obtained the following results. methylene blue diluted 1/1 million destroyed virus in sunlight in less than 30 seconds, and in diffused daylight within 2 minutes. After exposure to the action of diffused daylight for 1 hour, virus was completely destroyed by methylene blue *1/20 million, sanoflavine and trypaflavine 1/50 million. In the dark, these dyes* diluted 1/1,000 to 1/1 million were quite ineffective. Herzberg (1933 *b*) later studied the photodynamic effect of
Perdrau and Todd (1933) also
by methylene blue (1/100,000)
(1936) who report that vaccinia virus is destroyed by the photodynamic effect of methylene blue, toluidine blue, methylene green, thionin, and crystal violet in certain dilutions

In vivo the activity of certain thiacines has also been demonstrated Thus, rabbits were inoculated dermally with lymph and 1 to 2 hours later thiacine solution injected intravenously, the animals then being exposed to sunlight for an hour. No pustules formed in these animals (Herzberg, 1931 *a*, *b*).

Herzberg (1933 *b*) found that no lesions developed after scarification if methylene blue was given intravenously up to 24 hours later, and ultraviolet or sunlight irradiation carried out

Perdrau and Todd (1933) applied virus to the scarified skin of the rabbit, methylene blue was administered intravenously, and irradiation applied. There was no reaction if the dye was given up to 18 hours after the virus. Experiments were also carried out on the cornea, which was scarified with virus, the conjunctival sac then being washed out with dye. This treatment prevented the onset of keratitis if given up to 4 hours after the virus

Perdrau and Todd (1936) studied the time taken for virus to penetrate the cells of the host, after inoculation into the rabbit's testis At varying times after injection the testes were removed, suspensions prepared and exposed to the photodynamic effect of methylene blue They found that virus was not protected from the photodynamic effect until after 10½ hours of *in vivo* contact. Presumably, therefore, this time was required for the virus to penetrate the host's cells.

CHAPTER XXXIII

THE VARIOLA-VACCINIA VIRUS: IMMUNITY

A. EXPERIMENTAL AND THEORETICAL ASPECTS

1. The Antigenic Structure of Vaccinia Virus

OF RECENT YEARS very great advances have been made in our knowledge of the antigenic structure of vaccinia virus Much of this advance dates from Gordon's demonstration that flocculation occurs when a suspension of variola scabs is mixed with antivaccinial serum Next, it was found by the work of Tulloch and Craigie that vaccinia virus treated by Seitz filtration can be separated into 2 moieties, the one contains the elementary bodies and is not markedly flocculable by antiserum, while the other moiety is noninfective but markedly flocculable Later, it was found that elementary bodies contain antigenic components, known as L (labile) and S (stable). LS antigen is found in considerable amount in suspensions of dermal pulp. Independent L and S antibodies develop when LS antigen is inoculated into animals When antivaccinial serum is mixed with elementary bodies, then agglutination occurs, but if the serum is mixed with a suspension, then flocculation occurs, the same LS antigen and antibody being concerned in both reactions.

This subject of the antigenic structure of vaccinia virus has been studied by numerous authors (Burgess, Craigie, and Tulloch, 1929,[1] Craigie and Tulloch, 1931; Tulloch, 1931–2, Craigie, 1932 *a*, *b*, 1933, 1934, Smith, 1932, Craigie and Wishart, 1934 *a*, *b*, 1935, 1936 *a*, *b*, *c*, Salaman, 1934, 1937, 1938, Ch'en, 1934–5, Parker and Rivers, 1935, 1937, Wishart and Craigie, 1936, Smadel and Wall, 1937, Amies, 1938, Parker, 1938 *a*).

The effect of Seitz filtration on virus suspensions.

Suspensions of vaccinia virus can be separated into 2 moieties by the following method (Tulloch, 1931–2): The material is filtered through a Seitz EK disk, thus yielding a filtrate which is flocculable but noninfective, sometimes it is necessary to use double disk filtration to obtain a filtrate which is noninfective. Next, the disk is washed to remove any of the flocculable material, then it is reversed, and washing is carried out in the opposite direction. This operation yields a material which is infective, but not markedly flocculable, and contains the elementary bodies.

The LS antigen: work of Craigie and Wishart.

Craigie and Wishart have shown that various suspensions of vaccinia virus contain an antigen known as LS which is composed of 2 components L and S The LS *antigen is known as the specific flocculable* (precipitable) substance or the specific filtrable substance

The L antigenic component The antigenic properties are destroyed at 70° C., but resist the action of ether, L antigenic component is precipitated by CO_2 (Craigie and Wishart, 1936 *c*, Wishart and Craigie, 1936)

The S antigenic component. This component withstands 70° C.; its antigenic power is considerably lowered by steaming (Craigie and Wishart, 1936 *c*), but is unaffected at 90° C (Wishart and Craigie, 1936).

Certain authors, apart from Craigie and his coworkers, have isolated heat-stable antigenic components from vaccinia-infected tissues

Smith (1932) has isolated a thermostable substance from extracts of a testicular strain of vaccinia which he suggests is the same as Craigie's factor Salaman (1934)

[1] References are appended at the conclusion of Ch XXXV, p 403 et seq

and Rule, 1946; Fasquelle, 1947). Gohar and Bashatli (1946), however, claimed that if rabbits were treated with penicillin shortly after inoculation, only trivial lesions appeared.

Groupé and Rake (1947) working with eggs demonstrated an antibiotic effect with commercial, but not pure penicillin.

Streptomycin is also useless (Kolmer and Rule, 1946)

THE EFFECT OF CHEMICAL AGENTS ON VACCINIA VIRUS

Numerous chemical agents have been examined for a sterilizing effect on vaccinia virus and the following substances have been found to destroy it in varying times

Hydrochloric acid (N/1,000)	Gordon (1925)
Caustic soda (N/500)	Gordon
Ethyl, and methyl alcohol	Gordon, Yaoi and Kasai (1931 *b*)
Acetone	Gordon, Yaoi and Kasai
Izal and oil of cloves	Gordon
Potassium permanganate (1/100,000)	Gordon
Hydrogen peroxide	Gordon, Yaoi and Kasai
Camphor and thymol	Yaoi and Kasai
Hydroxylamine and hydrazine	Yaoi and Kasai
Sodium amalgam	Yaoi and Kasai
Sodium hydrosulfite	Yaoi and Kasai
Chloroform and ether	Yaoi and Kasai, Duran-Reynals (1928 *b*)
Quinine and optochin	Yaoi and Kasai
Iodine	Noguchi (1918), Yaoi and Kasai
Antiformin (1/10,000)	Yaoi and Kasai
Phenol	Noguchi (1918), Yaoi and Kasai (1930)
Bile	Courtois (1935)
Mercuric chloride	Beattie and Baldwin (1933-4)
Ascorbic acid	Kligler and Bernkopf (1937)
Potassium salicylate	Cooke and Best (1941)
"Liquor antisepticus" and lysol	Dunham and MacNeal (1942, 1942-3)

Though formalin appears to destroy virus, e g, in brain tissue, virus can be reactivated by dialysis or trypsinization (Galli and Vieuchange, 1939 *a*, *b*) However, if the suspension of virus is purified, and freed of tissue, virus cannot be reactivated (Levaditi and Reinié, 1939 *c*, Vieuchange and Galli, 1939 *b*)

Vaccinia is destroyed by glycerol more quickly than is herpes (Levaditi, 1941 *b*, Gohar and Bashatli, 1946)

THE EFFECT OF CERTAIN BIOLOGICAL AGENTS ON VACCINIA VIRUS

1. Beard and Rous (1938) have shown that the infectivity of vaccinia is largely suppressed when it is mixed with suspensions of living Kupffer cells or clasmatocytes, and then injected intradermally Such suppression of infectivity is not, however, due to the production of antiviral substances by these cells, for the virus proliferates in tissue cultures of Kupffer cells

2 Hirano (1931 *b*) found that vaccinia was strongly resistant to trypsin, was not digested by diastase, but was readily digested by lipase Pirie (1935) also found virus to resist trypsin, but it was destroyed by fatty acids and lecithin

3 It has been claimed that in the presence of complement, vaccinial antiserum acts on the virus so as to liberate a toxin, which produces a rash on intracutaneous inoculation of children (Brokman et al, 1930 *a*, Gastinel et al, 1931)

4 Vaccinia is not destroyed by the virus-inactivating agent present in human nasal secretion, or by saponin, or sodium desoxycholate (Burnet, Lush, and Jackson, 1939, Burnet and Lush, 1940)

serum, then flocculation occurs, for here the LS components are in solution (see in particular Craigie and Wishart, 1936 *c*, Wishart and Craigie, 1936). In short, the serological reaction resulting when vaccinial (or variolar) antigen is mixed with

ion occurs
ince; fixa-
tion also occurs between immune sera and elementary bodies (Craigie and Wishart, 1934 *b*, Finlayson, 1935).

Degradation of LS The nature of LS.

investigators now enables us to visualize fairly

covered with S antigen they behave like EB's in electrophoresis experiments, the mobility of EB's corresponds to that found for the S antigen (Shedlovsky and Smadel, 1940, Smadel *et al.*, 1940).

2 Electrophoretic experiments on virus-free filtrates of suspensions of dermal pulp show all LS activity to be associated with a single component This LS antigen precipitates to equal titer with optimal amounts of L and S antibody, and is completely removed from solution by adsorption with either. The LS antigen appears to be a protein with 2 antigenically distinct parts, L and S Heating modifies the L portion, so that it no longer precipitates with L antibody, but this "degraded" antigen still combines with L antibody to inhibit precipitation, it still precipitates with S antibody. This substance is known as L'S.

Treatment with heat and dilute alkali modifies S, so that it combines with S antibody and inhibits precipitation, but this antigen does not precipitate with S

L are
activi-
e sero-

ith the L and S antigens is present in one protein molecule This conception is slightly different from that of Craigie and Wishart, who considered the two antigens as ordinarily occurring in the form of a complex which could be dissociated (Smadel, Lavin, and Dubos, 1940, Shedlovsky and Smadel, 1942, Smadel and Rivers, 1942,

943) studied LS by electrophoresis, and that *LS is homogeneous, and has an iso-* rtial specific volume is 0 72 cc/gm, and its diffusion constant $1\,50\times10^{-7}$ cm²/sec The sedimentation constant is 6 35 at 20° C; the MW is 214,000. L'S and L"S' are homogeneous electrophoretically, but not in the centrifuge, L"S' being very polydisperse.

4 Smadel, Hoagland, and Shedlovsky (1943) found that the LS antigen contains 15 8 per cent N and 50 6 per cent. C, and is of protein nature The produce LS" contains the same amount of N as the native substance, but unlike LS, forms needle-shaped crystals

NP antigen.

Alkaline extracts of vaccinial EB's contain a nucleoprotein antigen present in amounts equivalent to 40–50 per cent. of the weight of EB's This antigen is heat-stable S antisera prepared by immunizing rabbits with heat-inactivated EB's also contain NP antibodies (Smadel, Rivers, and Hoagland, 1942).

2. The Precipitation Reaction

Precipitation (flocculation) can be elicited between vaccinial extracts or Seitz filtrates and antiserum, and early work on this reaction was carried out by various

obtained a similar substance from the skin crusts of dermal vaccinia. Ch'en (1934–5) claims to have isolated a polysaccharide substance which he regards as a haptene and a specific product of the vaccinia virus. Commenting on this work, Craigie and Wishart (1936 *c*) suggest that Ch'en's carbohydrate is a polysaccharide haptene of the S component of the LS antigen.

Parker and Rivers (1937) and Parker (1938 *a*) isolated a stable serologically active substance from tissues infected with vaccinia, which is probably identical with the S factor.

The source of LS antigen.

Most of Craigie and Wishart's work has been carried out with a dermal strain of virus "CL," but other strains, including testicular ones, can yield the LS antigen.

LS antigen is demonstrated most readily in suspensions of dermal pulp, prepared in the rabbit, calf, or guinea-pig (Craigie and Wishart, 1935); it occurs also in testicular pulp, and has been found in virus grown in tissue culture (Ch'en, 1934–5), and in the fertile egg (Smadel and Wall, 1937). Although LS is so readily demonstrated i [illegible] or whether it [illegible] necessary to demonstrate its presence (Craigie and Wishart, 1936 *c*).

LS antigen also dissociates from elementary bodies *in vitro;* the elementary bodies are still highly infective after this dissociation ceases (Craigie and Wishart, 1936 *b*). Further, these authors reported that elementary bodies dissociate 20 to 50 times less LS than does the volume of fresh pulp from which they are prepared.

Craigie and Wishart (1936 *b*) discuss the question whether the L and S antigenic components are produced within the virus particles, or whether they are formed outside. If the latter, it may be by the interaction of substances secreted by the virus with constituents of the infected cell. As elementary bodies dissociate much less LS than does the vaccine pulp from which these elementary bodies are prepared, they suggest that vaccinia virus exists in two forms. First, resting elementary bodies in which the metabolic activity is very low (see also p. 338), here there is little dissociation of LS. Second, in the intracellular or rapidly proliferative form, where there is much dissociation, they suggest that LS is elaborated by the virus in its active form. When the elementary bodies are separated from the cells, then LS is adsorbed on their surface and is not dissociated.

Additional evidence that LS is formed from the virus itself, and not from the host as was tentatively suggested by Sabin (1935 *b*), is afforded by the demonstration of LS in so many sources (*vide supra*).

Preparations of LS antiserum

It has been found that sera prepared in rabbits by inoculation of partly purified elementary bodies, or of Seitz filtrates of lapine, contain 2 agglutinins, acting on the L and S antigenic components respectively (Craigie, 1934, Craigie and Wishart, 1934 *a*, 1936 *c*, Wishart and Craigie, 1936).

Specific sera, to react only with either the L or the S antigenic factors, have also been prepared, and used to titrate the content of various vaccinial preparations in these components (Craigie and Wishart, 1935).

Both L and S antibodies are concerned in the agglutination or flocculation of vaccinial elementary bodies or extracts.

The rôle of LS in the serological reactions of vaccinia.

Both the agglutination and flocculation tests (described below) are dependent on the interaction of LS antigen and antibody. If pure washed elementary bodies, free of all flocculable substances, are treated with antiserum, then agglutination occurs. In this case the antigenic components are adsorbed on the surface of the elementary bodies. However, if a suspension of vaccine pulp is treated with anti-

serum, then flocculation occurs, for here the LS components are in solution (see in particular Craigie and Wishart, 1936 c, Wishart and Craigie, 1936). In short, the serological reaction resulting when vaccinial (or variolar) antigen is mixed with antiserum depends on the physical state of the antigen

1934 b, Finlayson, 1935).

Degradation of LS: The nature of LS.

Work by an American group of investigators now enables us to visualize fairly clearly the nature of the LS antigen

1. When collodion particles are covered with S antigen they behave like EB's in electrophoresis experiments, the mobility of EB's corresponds to that found for the S antigen (Shedlovsky and Smadel, 1940, Smadel *et al.*, 1940).

2 Electrophoretic experiments on virus-free filtrates of suspensions of dermal

with S antibody This substance is known as L'S

Treatment with heat and dilute alkali modifies S, so that it combines with S antibody and inhibits precipitation, but this antigen does not precipitate with S

These experiments indicate that all the serological activity associated with the L and S antigens is present in one protein molecule This conception is slightly different from that of Craigie and Wishart, who considered the two antigens as ordinarily occurring in the form of a complex which could be dissociated (Smadel, Lavin, and Dubos, 1940, Shedlovsky and Smadel, 1942, Smadel and Rivers, 1942, Smadel, Hoagland, and Shedlovsky, 1943)

3. Shedlovsky, Rothen, and Smadel (1943) studied LS by electrophoresis, and in the analytical centrifuge They found that LS is homogeneous, and has an isoelectric point at pH 4.8 At 4° C the partial specific volume is 0 72 cc/gm, and its diffusion constant $1\ 50 \times 10^{-7}$ cm²/sec The sedimentation constant is 6.35 at 20° C; the MW is 214,000 L'S and L"S' are homogeneous electrophoretically, but not in the centrifuge, L"S' being very polydisperse

4. Smadel, Hoagland, and Shedlovsky (1943) found that the LS antigen contains 15 8 per cent. N and 50 6 per cent. C, and is of protein nature. The produce LS" contains the same amount of N as the native substance, but unlike LS, forms needle-shaped crystals

NP antigen.

Alkaline extracts of vaccinial EB's contain a nucleoprotein antigen present in amounts equivalent to 40–50 per cent of the weight of EB's. This antigen is heat-stable S antisera prepared by immunizing rabbits with heat-inactivated EB's also contain NP antibodies (Smadel, Rivers, and Hoagland, 1942).

2. The Precipitation Reaction

Precipitation (flocculation) can be elicited between vaccinial extracts or Seitz filtrates and antiserum, and early work on this reaction was carried out by various

persons (Tanaka, 1902, Freyer, 1904, Tomarkin and Suárez, 1917, Torikata, 1917, Havens and Mayfield, 1931). Of special interest, in view of later work on the S and other heat-stable substances, was Torikata's demonstration that a boiled filtrate of calf lymph contained a heat-stable antigen that stimulated the development of precipitins on injection. Tomarkin and Suárez also stressed the value of heated extract in eliciting the precipitin reaction.

Recent work (see above) has shown that the flocculation reaction with crude suspensions is dependent on 2 distinct factors. First, a precipitin reaction with vaccinial antigen in solution; and second, an agglutination reaction in which the elementary bodies only are concerned. The rôle of the LS antigen in this reaction has already been discussed The precipitin reaction is of great value in the diagnosis of smallpox and is discussed elsewhere (see end of chapter).

3. The Agglutination Reaction

The injection of elementary bodies, or cruder virus-containing suspensions, into rabbits stimulates the development [illegible] hen, 1913, 1924, Ledingham, 1931, 1932, 1933, Parke [illegible] (1932), for instance, showed that if rabbits were [illegible] he shaved back, then agglutinins for elementary bodies developed in the serum.

Craigie (1933) showed that when previously vaccinated rabbits were inoculated with killed elementary bodies, there was an increase in agglutinins similar to that produced by living elementary bodies. He found that elementary bodies washed free of the flocculable substance could be used to yield an antigen for macroscopic tests when diluted 1, 20 to 1/50

It has been calculated that 1.95×10^8 particles per c c of suspension are needed to produce visible agglutination (Parker and Rivers, 1936 *b*).

Recent work has shown that the LS antibody in antivaccinial serum acts on the LS antigen to cause agglutination.

4. Inhibitory Effects in the Precipitation and Agglutination Reactions

Craigie (1932 *b*) studied these effects and reported as follows·

(*a*) The agglutination of vaccinial elementary bodies by immune serum at 50° to 55° C. is interfered with if fresh serum is used. Normal serum exerts a similar inhibiting effect.

(*b*) A similar inhibition affects the Seitz filtrable flocculable substance in the precipitin test

(*c*) This inhibiting effect is developed in fresh serum when heated by itself for 5 to 10 minutes at 56° C. It is also developed when fresh serum and antigen are incubated together at the same temperature.

(*d*) Immune serum to be used in agglutination and precipitation tests must be heated alone at 56° C. for 45 minutes to destroy this inhibitory effect.

(*e*) The inhibitory effect is not destroyed if antigen and serum are heated together at 56° C, even for 16 hours

5. The Complement Fixation Reaction

Many authors have shown that complement may be fixed by vaccinial lymph or infected tissue extracts in the presence of immune sera (Jobling, 1906, Beintker, 1908, Kolmer, 1916, Gordon, 1925, Gildemeister and Heuer, 1927 *b*, Bedson and Bland, 1929 *b*, Gilmore, 1931, Nakajima, 1932, Thompson, Hazen, and Buchbinder, 1932, Parker and Muckenfuss, 1933, Parker, 1934, Myers and Chapman, 1937, Weil and Gall, 1940, Rice, 1946)

Various materials may be used as a source of antigen in the complement fixation test, for example vaccinial dermal lymph, suspensions of infected testis, or brain (fresh, desiccated, or glycerolated), cocto-antigen, suspensions of infected

chick chorio-allantoic membranes, infected tissue cultures, and variolar vesicle or pustule contents. It has also been shown that complement is fixed by the interaction of antiserum with the elementary bodies or the Seitz filtrable moiety of vaccinia (Craigie and Wishart, 1934 *b*, Finlayson, 1935).

Complement fixing antibodies usually appear within 7 to 10 days of infection and persist for several months. The test is used in the serological diagnosis of smallpox, and further descriptions are given at the end of the chapter.

6. The Virus Neutralizing Test

It appears that normal whole blood or serum has some degree of virus neutralizing action (Douglas and Smith, 1930, Ramon *et al.*, 1942 *a*).

A number of workers has demonstrated that the sera of immunized animals and convalescent cases of smallpox or vaccinia have a neutralizing effect *in vitro* on preparations of virus (Sternberg, 1892, 1896, Béclère, Chambon, and Ménard, 1899, Camus, 1908, 1909, 1917, Kelsch *et al*, 1908, Teissier and Gastinel, 1912 *b*, Gins, 1916 *b*, Sato, 1921, Matsuda, 1924, Gins and Iwanoff, 1928, Schultz, Bullock, and Lawrence, 1928, Yaoi, 1935 *a*, 1937, Plotz, 1938 *c*). Gordon (1925) showed that the antibody was stable at 55° C, and that complement was not necessary. Béclère, Chambon, and Ménard (1899) showed that antibody was associated with globulin

The virus neutralizing antibody usually appears a few days after inoculation in rabbits, and may persist for 2½ years, longer than the duration of agglutinins or precipitins (Morgan and Olitsky, 1940). Thompson and Buchbinder (1931) detected a well-marked correlation between the titers of the flocculating and virus neutralizing antibodies, on occasions, however, the neutralizing antibody titer might be high while the flocculating titer was low Other workers have also drawn attention to a lack of correlation between neutralizing and agglutinating antibodies (Levaditi and Reinié, 1939 *b*, Levaditi *et al*, 1940 *b*).

Virus neutralizing antibodies can be titrated in various ways Haagen (1936 *a*) injected serum-virus mixtures cerebrally in mice (see also Bronson and Parker, 1941). Antibodies can be titrated by reduction in the number of vaccinial or variolar lesions produced on the chorio-allantois (Keogh, 1936, Blattner, Heys, and Gol- [illegible] *b*). Alternatively, serum-virus mixtures may [illegible] it's back (Loutit and McClean, 1945). Led- [illegible] have both devised methods based on rabbit titration.

Salaman (1938) believes that, under constant conditions, a given quantity of antiserum inactivates a constant percentage of any dose of virus upon which it acts Parker (1939) found that when the concentration of serum is held constant, the quantity of virus neutralized depends on the amount added Further, the proportion of virus neutralized by a given concentration of serum increases as more concentrated suspensions of elementary bodies are used. When the immune serum is diluted, the serum becomes relatively more efficient in neutralizing the virus, although the actual quantity of virus neutralized is diminished.

7. The *in vitro* Interaction between Vaccinia Virus and Antiserum

As has been stated, virus neutralizing antibodies can be demonstrated readily in immune sera The important question arises as to the state of the virus in serum-virus mixtures that appear neutral, for example, on application to the rabbit's skin Numerous workers have demonstrated the presence of active virus in such supposedly neutral mixtures, using the following methods for reactivation

Injection of a mixture, apparently neutral when injected dermally, into a more susceptible tissue such as brain, testis, or chorio-allantois (Andrewes, 1928, Lépine, 1930, Fairbrother, 1932, Myers and Chapman, 1937, Vieuchange, 1938 *b*, Vieuchange and Mesrobeanu, 1938)

Filtration (Andrewes, 1928, Sabin, 1935 *a*)

Adsorbing with kaolin (Andrewes, 1928, Goyal, 1935).
Simple dilution (Andrewes, 1928; Long and Olitsky, 1930, Goyal, 1935; Keogh, 1936).
Cataphoresis (Lépine, 1930, Long and Olitsky, *1930*).
Centrifugation (Sabin, 1935 *a*, Magrassi and Hallauer, 1936).
Addition of testicul[illegible]
However, even in [illegible] that some portion of the virus had been [illegible] ..longed interaction, a specific union does occur, *and that virus* and serum cannot then be readily dissociated (Andrewes, 1930, McKinnon, 1930; Craigie and Tulloch, 1931; Gilmore, 1931; Fairbrother, 1932; Vieuchange and *Stamatin, 1938*).

8. Allergy

The skin of animals becomes allergic on recovery from infection, and responds to reinoculation by an accelerated *reaction (see, e g,* Thompson, 1930 *b*, Dienes and Naterman, 1937; Plotz, 1938 *c*; Levaditi *et al.*, 1940 *a*) Henseval (1919 *a*) found that no allergic response was elicited on reinoculation if the area of primary infection *was excised after* 3 days, when it was excised after 6 days, a typical allergic response developed on revaccination. Force and Beckwith (1915) noted a severe reaction *when vaccinia*-infected rabbits were inoculated with variolous material.

Sensitization may be induced by inoculations of killed vaccinia (Gastinel and Fasquelle, 1943 *c*).

The allergic response has been elicited by a reinoculation of boiled (variola) virus (Blaxall, *1923*). Buchbinder (1932) could not demonstrate any specific contraction of the uterine muscle of sensitized guinea-pigs when exposed to vaccinia *in vitro*.

The state of allergy may develop independently of resistance to vaccinial infection, e g, after sensitization induced by killed virus (Gastinel and Fasquelle, *1943 a, c*).

9. The Preparation of Antivaccinial Sera

Antivaccinial sera, for use in serological tests, or for conferring passive immunity, are usually prepared by repeated injections of rabbits with virus Antisera to the L or the S antigenic components can be separately prepared, but sera obtained by inoculation of partially purified elementary bodies or vaccinia-infected suspensions contain both of the antibodies. Representative methods are described in detail elsewhere (see p 371). Immune sera have also been prepared by injection of horses (Béclère, Chambon, and Ménard, 1899, Ledingham, Morgan, and Petrie, 1931, Bridré and Bardach, 1938, Cesari and Boquet, 1938, Ramon, Boquet, and Richou, 1942, Ramon *et al*, 1942 *b*), buffaloes (Pandit *et al*, 1931–2), goats (Yaoi, 1935 *a*, 1936 *b*), and guinea-pigs (Bedson and Bland, *1929 b*).

Findlay (1931) found that in antivaccinial rabbit sera the immune body responsible for the protective action, flocculation, and action stimulating the "phagocytosis" of virus was associated with the globulin

In the method described by McKinnon and Knowles (1931) the virucidal antibody was obtained in approximately fivefold concentration in the pseudoglobulin, the euglobulin containing only a small amount.

Antivaccinial serum may be titrated most easily by estimating the virus neutralizing antibody (p. 363)

Site of production of immune bodies Only a few observations bearing on this point have been reported For example, the importance of the spleen has been suggested, as it has been claimed that immune bodies develop in tissue cultures of spleen infected with vaccinia (Kimura and Fujisawa, *1931*)

The rôle of the reticulo-endothelial system was investigated by Mundel and Zurukzoglu (1935), who found that virucidins appeared more quickly in the sera

of rabbits in which "blockade" had been carried out with trypan blue than in controls.

McMaster and Kidd (1937) have shown that an antiviral substance is produced in the regional lymph glands which drain the area of skin injected with virus For example, after inoculation of the skin of the rabbit's ears, virus was carried to the regional nodes, and an antiviral substance could be demonstrated in a few days in greater concentration in the nodes of the injected side than in the serum. After inoculation in the skin, virus neutralizing antibody may be produced locally (Vieuchange and Galli, 1939 *a*, Hartley, 1940, Vieuchange, 1940).

Extracts of spleen, lymph glands, and testes of immunized animals neutralize vaccinia virus (Douglas and Smith, 1930, Vieuchange, 1937).

10. Antibody Absorption

It has been shown that vaccinial antigen can absorb specific antibody from immune serum.

1. For instance, Gordon (1925) showed that flocculating antibodies could be absorbed from antiserum by saturation with vaccinia virus. He found that this treatment removed antibody for both vaccinia and variola

2. Smith (1930) showed that an emulsion of vaccinia-infected testis removed homologous antibody from immune serum further, if the testis emulsion was heated at 58° C. to 60° C. for 1 hour, the power of absorbing antibody was greatly reduced

3 Salaman (1937) has carried out experiments on the absorption of immune sera, with the following results

(*a*) Absorption of antiserum with elementary bodies removes all virus neutralizing antibody, but absorption with specific precipitating substance does not.

(*b*) Absorption with elementary bodies removes all agglutinins, but not all precipitins. Absorption with specific precipitating substance removes all precipitins, but not all agglutinins.

(*c*) Virus neutralizing power, but no agglutinin or precipitin, may be found in certain sera obtained 3 to 4 months after vaccination. This virus neutralizing antibody can be absorbed by elementary bodies, but not by specific precipitating substance.

4. S antibody can be absorbed from LS antisera by treatment with heat-stable vaccinial extracts (Parker and Rivers, 1937).

11. Passive Immunity

Various observers have shown that injections of antiserum given shortly before virus, or in certain cases up to 2 days later, may prevent or modify a local or generalized vaccinial infection, the serum may be injected intravenously, subcutaneously, or at the site of virus inoculation, rabbits, mice, and monkeys have been used (Raynaud, 1877, Hlava and Honl, 1895, Béclère, Chambon, and Ménard, 1896, Camus, 1912 *a*, *b*, Gordon, 1925, Hunt and Falk, 1927, Andrewes, 1929, Craigie and Tulloch, 1931, Ledingham, Morgan, and Petrie, 1931, Pandit *et al*, 1931–2, Yaoi, 1935 *a*, 1936 *b*, Schultz and Hartley, 1936, Andersen, 1937 *b*, Iguchi, 1939 *b*, Vieuchange, 1939 *a*, *b*, Green and Parker, 1942)

It has been claimed that maternal immunity can be transmitted to the offspring (see, e g, Sato, 1921, Nelson, 1932, 1934, Andersen, 1937 *c*).

Nelson (1932) concluded that in the pig the placenta was largely impermeable to protective substances, which were probably transmitted by the colostrum Later (1934), he found that the immunity acquired by sucklings began to wane during the 2nd month, and was almost absent at the end of the 3rd.

Andersen (1937 *c*) studied this question in the young of rabbits which had been vaccinated before pregnancy He found that the young showed virus neutralizing serum antibodies 24 to 48 hours after birth, and that these persisted for at least 3

weeks Correspondingly, these animals were resistant to vaccination from 24 hours to 3 weeks after birth After about 3 months he found that both resistance to infection and antibodies were no longer present. He believed that the state of resistance was directly dependent on the passage of antibodies to the offspring from the mother.

12. Active Immunization

With living virus.

Monkeys become resistant to reinoculation on recovery from infection with variola or alastrim (see, e.g, Horgan and Haseeb, 1939), and the same has been reported for mice (Nelson, 1940 *a*).

It has, of course, been known for a long time that on recovery from vaccinial infection, experimental animals become immune to reinfection. Animals may be immunized by applying living virus to the scarified skin, by inoculating it intra- or sub-cutaneously, or by intraperitoneal injection. The intravenous route, in distinction to the dermal, has been especially recommended for the immunization of rabbits (Kelsch, Camus, and Tanon, 1908, Teissier, Duvoir, and Gastinel, 1912, Camus, 1915, 1916, Myers and Chapman, 1937) It was, in fact, realized early that, to produce immunity, it was not necessary for an eruption to follow the immunizing injection (see, e.g., Raynaud, 1878, Béclère, Chambon, and Ménard, 1898, 1899) Immunity can also be produced by intracerebral, intra-ocular, intratracheal, and intrapulmonary injection, and by instillation of the external auditory canal, conjunctiva, and nose (Calmette and Guérin, 1901, Gordon, 1925, Gins, 1929). Subcutaneous injection in the chin has been specially recommended in rabbits (Yaoi and Arakawa, 1939 *b*)

Resistance to intracutaneous injection is most readily acquired, being usually evident after the 4th day, and pronounced after 10 days, after 100 days the immunity is very much reduced, although some degree may persist for 3 years (see e g, Camus, 1915, Gordon, 1925, Morgan and Olitsky, 1940) It is more difficult to immunize rabbits against infection by routes such as the cornea, testis, or brain, and for this purpose large and repeated doses are usually required Nevertheless, certain workers have reported that resistance to cerebral challenge may develop quickly, even in 4–6 days after dermal or other inoculation (Levaditi and Nicolau, 1923 *a*, Rivers, Sprunt, and Berry, 1933, Levaditi *et al*, 1939 *c*, Gastinel and Fasquelle, 1943 *b*) Mice have also been rendered resistant to cerebral challenge by injections given cerebrally, nasally, or subcutaneously (Iguchi, 1939 *a*, *b*, Bronson and Parker, 1944)

With physically or chemically inactivated virus.

Numerous experiments have been carried out using inactivated preparations of vaccinia virus Immune bodies and resistance to infection develop after such inoculations

(*a*) *Using heated virus* Various investigators have experimented with the effect of heat on the antigenic potency of vaccinia (see, e g, Supfle, 1909, Knoepfelmacher, 1915, Henseval, 1919 *a*, Murata, 1924, Nakagawa, 1924 *a*, *b*, 1925, Gordon, 1925, Hunt and Falk, 1927, Bland, 1932, Kligler and Bernkopf, 1935–6, 1937) Resistance to infection can be produced by injection of virus heated to not more than 65° C. Torikata (1917), however, claimed to have induced immunity with boiled antigen

(*b*) *Using chemically inactivated virus* A number of workers has tested the effect of *formol* on the immunizing quality of vaccinia Hilgers (1931–2) found that formol-inactivated virus was most erratic and not to be relied upon to produce immunity Many other workers claim, however, to have immunized rabbits successfully with formolized virus (Hunt and Falk, 1927, Iwanoff, 1927, Kraus, 1929–

30, Craigie, 1934, Kligler and Bernkopf, 1935–6, Bernkopf and Kligler, 1937; Weil and Gall, 1940). Bland (1932) found that rabbits were difficult to immunize, whereas guinea-pigs were more readily rendered resistant

Parker and Rivers (1936 *a*) carried out an important study with formol-inactivated elementary bodies. Their results were as follows (1) Antibodies, and a temporary degree of resistance to infection, can be produced in rabbits by several injections of elementary bodies rendered inactive by 0 3 per cent. formol. (2) Small repeated doses of elementary bodies are more efficient than single doses of larger amounts (3) The antigenic power of elementary bodies is almost completely destroyed by 10 per cent. formaldehyde or boiling for 2 hours

Phenol-treated virus has also been used for active immunization Gordon (1925), for example, found that phenolated virus provoked resistance to infection, and after a lapse of 100 days this was equal to that stimulated by living or attenuated virus Kraus (1929–30) also claims to have protected rabbits following injections of carbolized lymph Bland (1932) found that guinea-pigs were more readily immunized than rabbits Bigheri (1931), however, was not able to immunize rabbits by phenolated virus

Virus rendered noninfective by bile is still antigenic (Levaditi *et al*, 1940 *b*). Large doses of EB's inactivated with 50 per cent. alcohol are slightly antigenic (McClean, 1945).

(*c*) *Using physically treated virus* Vaccinia treated with ultraviolet light, alpha rays of radon, or supersonic waves is still antigenic (Kasahara and Ogata, 1939, Levaditi *et al*, 1940 *b*, Levaditi and Pérault, 1943).

The development of antibodies. Various workers have noted the development of antibodies after vaccination with inactivated virus Craigie (1934) carried out studies on the development of immune bodies after the inoculation of elementary bodies inactivated by heat, ether, or formol The elementary bodies used for inoculation were washed free of specific precipitable substance. In rabbits inoculated intravenously with these killed elementary bodies, agglutinins developed after the first injection, but no complement fixing properties until 2 to 3 had been given, and precipitins not until 3 were given He noted that formolized elementary bodies produced qualitatively a greater antibody response than heated elementary bodies, or living virus Subcutaneous or intradermal inoculation of formolized elementary bodies provoked poor antibody response.

Parker and Rivers (1936 *a*) found that the development of precipitins and agglutinins did not run parallel with that of neutralizing antibodies. Further, the development of antibodies did not necessarily indicate that the animal was strongly resistant to reinfection

Scalfi (1938) found that immune bodies to vaccinia developed on inoculation of splenic tissue from an infected animal, although this tissue contained no virus

With serum-virus mixtures.

It has been shown that mixtures of virus plus serum, although noninfective on intradermal inoculation, may nevertheless induce immunity, these injections may be given intradermally, by nasal instillation, or subcutaneous injection (e.g, Craigie and Tulloch, 1931, Findlay and Hindle, 1931, Rhoads, 1931). Fairbrother (1933) showed that a considerable resistance to infection might develop after intracerebral injection of mixtures of virus and antiserum that appeared to be noninfective intradermally (see also Iguchi, 1939 *b*)

By other means.

Duran-Reynals (1931) found that animals kept in the same room as those used for

stra

trates no immunization resulted. Yamada (1940) also claimed that virus-free filtrates were antigenic.

Armstrong (1932) claimed that mice immunized with diphtheria toxoid showed less severe reactions after intracerebral injection of vaccinia than did untreated animals. He also (1935) found that irritation of the rabbit's conjunctival sac by instillation of diphtheria toxin rendered the eye relatively resistant to infection with vaccinia.

13. The Mechanism underlying Resistance to Vaccinial Infection

The rôle of antibodies.

It is known that one of the properties of vaccinial antiserum is to cause a neutralization of virus, although it is questionable how stable a union is formed between serum and virus. Further, passive immunity can be conveyed by injecting vaccinial antisera into experimental animals (*vide supra*). It would thus seem probable that humoral immunity plays some part in the mechanism of resistance to infection. Andersen (1937 *c*) has supported this view from a study of neutralizing antibodies and resistance to infection in the young of vaccinated rabbits, he concluded that resistance was directly associated with serum antibodies

Magrassi and Muratori (1937), however, have published observations which tend to emphasize the relative unimportance of virus neutralizing serum antibodies They immunized rabbits by intradermal inoculations of virus plus antiserum, and found that virus neutralizing antibodies developed, and that the animals resisted injections into the skin, testis, or brain. Now, if the animals were immunized with formolized virus, very little resistance to reinfection was produced, but the virus neutralizing antibodies appeared, sometimes to as high a level as in animals treated with the virus-serum mixtures. They suggested that in the case of animals injected with the mixtures a latent infection developed, which had the effect of stimulating the appearance of tissue antibodies. After injections of formolized virus, however, no such latent infection developed, and although antibodies reacting *in vitro* could be demonstrated, true tissue immunity could not (see also Magrassi, 1937, Magrassi and Gregori, 1937).

The rôle of leukocytes.

Various authors have attributed some action to the leukocytes in the defense mechanism against vaccinia (Long and Olitsky, 1930, Fairbrother, 1933, Sabin, 1935 *c*) Fairbrother, for example, showed that immune blood had greater neutralizing properties than immune serum, he suggested that phagocytosis was an important defense mechanism of immune animals, as the effect of serum was enhanced by the addition of leukocytes.

Sabin showed that leukocytes may remove virus from the circulation although they may not destroy it

The persistence of virus in the immune animal.

Much interest was aroused by certain observations of Olitsky and Long (1929), who claimed to have recovered active virus by cataphoresis from the testes of animals long since convalescent from a cutaneous vaccination They suggested that resistance to vaccinia was connected with the persistence of virus in the body, and that there was no immunity without such persistence These results did not, however, receive confirmation at the hands of Herzberg (1931 *a*) or Pearce (1940)

The rôle of the cell.

Apart from any acquired insusceptibility, there is no doubt that some cells have an inherent degree of resistance to vaccinia virus, for example, larger numbers of EB's are needed to infect by the skin than by the brain

There appears to be some evidence that a local immunity can be developed in vaccinia. Thus, Camus (1912 *a*) found that the cornea could be immunized by instillation of antiserum in the conjunctival sac Immune corneae cannot be infected readily with vaccinia in tissue culture (Rivers, Haagen, and Muckenfuss, 1929 *c*) The question has been complicated by the finding of Stoel (1930–1) that although immune testis could be infected in tissue culture, immune spleen could not, he attributed this to the presence of macrophages and leukocytes in the splenic cultures Nevertheless, Florman and Enders (1942) found that virus multiplied in tissue cultures of monocytes from an immune rabbit.

Experiments have shown that virus may become fixed to the cells of a tissue culture, but cannot grow, if the culture is prepared with antiserum. If cells are first treated with serum and then inoculated, virus does not grow. It seems that antibody is fixed to the cell, and thus inhibits the entrance of virus, rather than becoming combined with it (Sabin, 1935 *b*, Magrassi and Hallauer, 1936, Vieuchange a
nomenon can be demon-
strated o
1939).

It is
e to vaccinia in the im-
mune an
bably leukocytes are all
concerned. Whatever the mechanism, immune tissue shows only slight changes and virus is rapidly eliminated on reinjection (see, e g , Force, Beattie, and Lucia, 1939).

B. PRACTICAL APPLICATIONS OF SEROLOGICAL REACTIONS IN HUMAN VACCINIA AND VARIOLA

The Precipitation Reaction

Tanaka (1902) showed that precipitation occurred when serous fluid from a case of variola was mixed and incubated with vaccinial lymph Thereafter there was little advance till Gordon (1925) showed that extracts of variolar scabs gave precipitation reactions with vaccinial antisera.

The precipitin test
smallpox by the work
in 1927 there were 152
and were tested for fl
The test was found to be of most value in confirming cases clinically typical, and in establishing a correct diagnosis in doubtful or actually unlikely cases The test was also shown to be of value in the diagnosis of cases of smallpox occurring in Dundee and elsewhere in 1928. The test was remarkably specific, in particular, chickenpox crusts gave a negative result. Tulloch (1934) has published a useful review of the work carried out on the serological diagnosis of smallpox. The technical details of the precipitation test in the diagnosis of smallpox are described at the end of this chapter.

Tomarkin and Suárez (1917) showed that the precipitating antibody developed in human sera after primary vaccination in about 10 days, persisting for a few months After revaccination, the antibody appeared in about 7 days Havens and Mayfield (1931) also recorded the presence of precipitins in the sera of vaccinated persons

Complement Fixation Reaction

Several early workers showed that complement fixation could be elicited using

variolar pustular exudate and variolar sera. Similar results were obtained by other early workers (e g Dahm, 1909, Xylander, 1909, Kryloff, 1911, Teissier and Gastinel, 1912 *a*, *b*, Klein, 1914, Konschegg, 1915).

Kolmer (1916) carried out valuable studies on the complement fixation reaction in vaccinia and smallpox. His results were as follows:

(a) Using scabs and vesicle contents of smallpox patients, he obtained positive fixation with the sera of vaccinated rabbits. This result was later confirmed by Gordon (1925).

(b) Using the sera of persons suffering from mild smallpox, he found that about 60 per cent. gave fixation with vaccinial or variolar antigens.

(c) Complement fixation was also obtained using mixtures of serum from persons previously vaccinated and vaccinial antigens.

It is thus evident both that complement fixing antibodies can be demonstrated in the sera of cases of variola or vaccinia, and that variolar scabs may be used to demonstrate fixation with animal antisera. A number of other workers have arrived at similar conclusions (Gilmore, 1931; Havens and Mayfield, 1932; Venkataraman, 1932–3, Parker and Muckenfuss, 1931–2, 1933, Parker, 1934).

Craigie and Wishart (1936 *a*) have strongly recommended the complement fixation test in variola. They state that the test is 8 to 10 times as sensitive as the flocculation test, and more easily carried out in routine laboratories, 0.008 gm. of clean crusts, or the total contents of 6 lesions, furnishes enough material to act as antigen. The antiserum should contain S antibody. The technique of this test is described in an appendix at the end of this chapter.

Downie (1946) found the CFT with vesicle fluid or crusts the most valuable single test; the material from 6 lesions may be sufficient. He also reported on the value of testing the patient's serum for complement fixing antibodies, using vaccinia antigen prepared in the rabbit, these antibodies may be present after the first week of illness.

The Virus Neutralizing Test

Virus neutralizing antibodies appear in the sera of cases of variola and of vaccinated persons (see, e.g., Béclère, Chambon, and Ménard, 1899, Schneider, 1923–4. Frisch, 1935, Greengard and Wolf, 1940, Blattner, Heys, and Gollub, 1943, Downie and Dumbell, 1947 *b*). They reach a high level about 16 days after the onset of variola (Blattner, Heys, and Gollub, 1943). Serum from vaccinated persons contains maximum antibody in the 3rd and 4th week (Loutit and McClean, 1945). Antibody may persist for a year or so.

APPENDIX

TECHNIQUE OF THE VARIOLA-VACCINIA FLOCCULATION AND COMPLEMENT FIXATION TESTS[1]

In the preparation of these instructions we wish to acknowledge the invaluable assistance of Professor W. J. Tulloch of Dundee, Scotland.

Reagents Required

1. The material to be investigated.
2. Variola, vaccinia, or lapine crusts to serve as a positive control.
3. Varicella crusts to serve as a negative control.
4. Lapine immune serum prepared by hyperimmunizing rabbits with extracts of vaccinia passaged in that animal species.
5. Normal rabbit serum.

The above reagents are required for the flocculation test, and for the complement fixation test the following additional materials are required:

6. Pooled guinea-pig complement.
7. Hemolytic system.

The control reagents, i.e., "2" and "3," are best stored in the form of dried crusts, preferably dried from the frozen state, while the material to be investigated consists either of crusts or of swabs impregnated with the contents of vesicles and allowed to dry.

[1] The CFT is preferred in routine diagnostic work owing to its greater sensitivity and easier technique. The flocculation test is now chiefly of academic interest.

Preparation of Antigen

To prepare an antigen for the tests, quantities up to 40 milligrams of dried crusts are triturated and 0.2 c.c. of 8.5 per cent NaCl solution added.

The crusts are allowed to macerate in this fluid for several hours at room temperature, when 1.8 c.c. of neutral distilled water is added and the extracts centrifuged at high speed for 15 to 20 minutes. The supernatant fluid so obtained represents a 1/50 extract of the crusts and constitutes the antigen. This extract can be suitably diluted for performing the tests.

In the case of swabs, the actual quantity of material available for extraction is, of course, unknown. In extracting these it is advisable that the complement fixation reaction, rather than the flocculation test, be used because of the greater delicacy of the former.

In extracting swabs, 0.2 c.c. of 8.5 per cent NaCl is put in a small tube, and in this the impregnated tip of the swab is extracted, as is done in the case of crusts.

Use of Antiserum containing Antibodies to the S Fraction of Craigie's LS Antigen

In immunizing rabbits for the preparation of diagnostic serum, the immunizing inoculum should consist of extracts that have been heated to 70° C. for 1 hour. This is done to insure that antibody to the S of the LS antigen, obtained from extracts of various material, will be present in the antiserum.

Such sera before use should be titrated against the S fraction of the antigen. This is advisable because the L fraction of the LS complex is unstable, and deterioration of the L fraction may occur in transit. If then diagnostic antiserum potent only in respect of the L fraction were used, false negative reactions would occur when extracts of such "deteriorated crusts" constituted the antigen for the test.

Sera to be used for these tests—both immune serum and normal control serum—should be inactivated by exposure to 56° C. for 45 minutes and stored without preservative.

It may be convenient to dry such sera from the frozen state and store *in vacuo*, if the product is only to be used infrequently, and if danger of contamination is feared. The dried sera are reconstituted when required by dissolving the dried powder in the requisite amount of distilled water. 1 gram of dried serum represents on an average 16 c.c. of the material in its fluid state.

Preliminary Titration of Antiserum

When the antiserum has been harvested it is titrated by the following method.

1/1,000 extract of lapine is prepared and is added to serial dilutions of serum. The range of dilutions of serum distributed is from 1/12.5 to 1/400, doubling the dilution at each tube, the volume being 0.25 c.c. To each of these is then added 0.25 c.c. of a 1/500 extract of lapine crusts, so giving a final range of concentrations of serum from 1/25 to 1/800 tested against an antigen consisting of a 1/1,000 extract of crusts.

An exactly similar series of control tubes with normal rabbit serum is set up at the same time.

The mixtures are kept at room temperature for 2 hours and then transferred to an incubator at 55° C., precautions being taken to prevent evaporation, for 16 hours. The results are then read, using an agglutinoscope to observe the flocculation.

The result of such a test is shown in the following table.

TABLE 13

Final dilutions of crust extract 1/1,000

Final dilutions of serum					
1/25	1/50	1/100	1/200	1/400	1/800
Immune rabbit serum					
++++	++++	++++	++	+	–
Normal rabbit serum					
–	–	–	–	–	–

++++ = marked flocculation easily visible to the naked eye, ++ = flocculation discernible without agglutinoscope, + = flocculation discernible only by agglutinoscope, – = no flocculation.

On this finding the titer of the serum would be accepted as 1/400.

Flocculation Test

In this test the antigens are employed in varying dilutions, while the serum is present in constant concentrations throughout, and the concentration of serum used is 10 times the titer as determined by preliminary examination.

1. Distribution of antigens.

Each antigen is diluted in a series of tubes to give concentrations of 1/125, 1/250, 1/500, 1/1,000 and 1/2,000, the volume per tube being 0 25 c c.

These are set up in duplicate so that to one series immune serum may be added, and to the other normal rabbit serum, to serve as a control

2. Dilutions of sera.

To each of the tubes of one series is now added 0 25 c.c. of immune serum diluted to 20 times the titer· in the case of a serum of the titer shown in the above table, this dilution would be 1/20. To each tube of the second series is added the same volume of normal rabbit serum but of twice that concentration, i e., 1/10

Mixture is insured by shaking, and all the tubes are kept at room temperature for 2 hours, after which they *are transferred to an incubator at 55° C for* 16 to 18 hours, precautions being taken to prevent evaporation.

The results are read in the same way as in the preliminary test shown in the previous table, and it must be emphasized that "zone reactions" are liable to be encountered It is for this reason that series of dilutions of antigens must be tested.

An example of the type of finding to be expected is shown in the following table

TABLE 14

Extracts of crusts tested	Immune rabbit serum final dilution 1/40 Dilutions of crust extracts				
	1/250	1/500	1/1,000	1/2,000	1/4,000
Lapine	+	+	+++	++++	+++
Test material	–	–	+	+++	++
Varicella	–	–	–	–	–
	Normal rabbit serum final dilution 1/20				
Lapine	–	–	–	–	–
Test material	–	–	–	–	–
Varicella	–	–	–	–	–

Complement Fixation Test

The complement fixation reaction is estimated by Craigie and Wishart (1936*a*) to be 8 or 10 times as sensitive as the flocculation reaction Its only disadvantage as compared with the flocculation test is that occasionally extracts of crusts exhibit anticomplementary qualities to an unexpected degree

The reagents are the same as those used in the flocculation test, but in addition complement and hemolytic system are also required.

For this test the complement should cons st of the pooled serum of several guinea-pigs, which is most conveniently prepared by exsiccation from the frozen state, being preserved *in vacuo* in sealed ampules

The hemolytic system consists of a 5 per cent suspension of washed sheep corpuscles sensitized with 5 minimum sensitizing doses of the requisite antiserum

Preliminary Titration of Antiserum

In this, as in the flocculation test, zone phenomena may be encountered Therefore, before being used for diagnostic reactions, immune serum should be titrated by exposing a series of dilutions thereof to a series of dilutions of known positive antigen In so doing, the dilution of antigen should be carried sufficiently far (1/16,000 to *1/32,000) to insure* that negative results are obtained at the lower limit of the series The serum is subsequently used in that concentration with which, in the test performed, the antigen gave

employed to con-

Diagnostic Complement Fixation Test

The hemolytic activity of the complement (m.h.d.) is determined as usual, and the following figures indicate the mixtures of antigen, antibody, and complement which are exposed to one another.

A. In Actual Test each tube contains

1. Antigen serial dilutions from 1/250 to 1/16,000 . . . . . 0.2 c.c.
2. Complement 3 m.h.d. . . . . 0.2 c.c.
3. Serum constant (optimum) dilution . . . . 0.1 c.c.

At the same time controls—"B" and "C"—designed to insure that neither the antigen nor the antiserum are anticomplementary, are put up.

B. Control of Antigen.

1. Antigen in serial dilutions as in A. . . . . 0.2 c.c.
2. Complement 1.5 m.h.d. . . . . 0.2 c.c.
3. Saline . . . . 0.1 c.c.

C. Control of Antiserum

This test is made in duplicate using 2, 1.5, 1, and 0.75 m.h.d. of complement in one series, the antiserum being present in the same concentration as that used in the test, while in the other the same volume of saline is introduced.

The procedure is shown in the following scheme

TABLE 15

Tube Number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Complement as in test, diluted 1/3	0.4	0.3	0.2	0.15	0.4	0.3	0.2	0.15
Saline	0.1	0.2	0.3	0.35	.	0.1	0.2	0.25
Serum as used in test					0.1	0.1	0.1	0.1

D. Incubation.

Tests and controls are incubated at 4° C. in the cold room for 14 to 16 hours, after which they are set at room temperature for 30 minutes, when 0.25 c.c. of hemolytic system is added to each tube.

The tests are transferred to a water bath at 37° C. and incubated for 30 minutes, when final readings are taken.

In positive reactions some of the range of concentrations of the antigen will give complete fixation of the 3 m.h.d. of complement, while in the cases of controls, lysis should be complete in all tubes containing 1.5 m.h.d. of that reagent.

In their original communication, Craigie and Wishart stress 2 points that are worthy of note

(*a*) The need for incubation at low temperature overnight to insure maximum sensitivity.

(*b*) The employment of the immune serum in concentrations known to give fixation with the stable S fraction of the antigen.

(In describing these diagnostic methods free use has been made of the original contributions of Craigie and Tulloch (1931) and of Craigie and Wishart (1936*a*), to which the reader is referred for further information.)

CHAPTER XXXIV

VACCINATION

Historical

IMMUNIZATION against smallpox originated in the East, where it was known that persons deliberately inoculated with material from a mild case developed a modified form of the disease. This practice of variolization was introduced into England by Lady Mary Wortley Montagu (1689–1762) In England the benefits of variolization were supported by Mead (1747),[1] and in America by the Mathers (see Singer, 1928). This practice was not free from risk, and some patients developed a virulent generalized attack and died. In more recent times, there is a record of leprosy being transmitted (Mora Roldán, 1932).

Sutton from 1764–1766 introduced the safer practice of variolization by arm-to-arm transfer (see McIntosh, 1930).

The next advance was the discovery by Jenner (1798) that material from the lesions of cowpox in man or cows could be inoculated artificially, and induce resistance to variolous material. By 1804 (see *Brit med J*, 1937, *2*, 675), millions of persons had been "vaccinated" with cowpox or "vaccinia" (the 2 viruses then being regarded as identical). Woodville and Pearson, attached to the Smallpox Hospital in London, adopted Jenner's method and vaccinated large numbers of persons by the arm-to-arm method They started with cowpox material, but their stock of lymph almost certainly became contaminated with variola The vaccinial lymph distributed by Woodville to foreign workers was probably chiefly variolous in origin, Jenner himself supplied various foreign observers (see *Med. Phys. J. 3*, *471*, *5*, *340*, *Baron*, *1*, *348*, *439*, *533*)

Vaccination was introduced shortly afterwards into other countries Sweden and Denmark in 1801, India in 1802, Egypt in 1827, following which there was a marked decline in the incidence of the disease

Vaccination Legislation

In England until the introduction of the National Health Service Act, vaccination legislation was governed by the 1898 Act, which although encouraging and making provision for free infant vaccination, contained a clause allowing conscientious objection. There is now no compulsory vaccination in England. The bulk of the lymph required in England was prepared in the Government Lymph Establishment at Colindale, which functioned from *1898–1946* (see *Fremlin, 1946*). Lymph is now prepared at the Lister Institute.

The vaccination laws in America vary from state to state (see Fowler, 1941, U.S Public Health Report, 1941) Investigators in America have canvassed parents to inquire into the incidence of vaccination (Breslow, Shalit, and Anderson, 1943, Gover and Yankev, 1946)

GENERAL PRINCIPLES IN THE PREPARATION OF CALF LYMPH

At a meeting of the Health Organization of the League of Nations at Geneva in August 1928, a number of experts (Aldershoff, Blaxall, Camus, Gins, Gordon, Gorth and Sobernheim) discussed current methods in use for the preparation and preservation of vaccine lymph The recommendations of this *Sub-Commission* were embodied in the text of a report (1928), containing a number of important facts relating to certain general principles involved in the manufacture of lymph, these

[1] References are appended at the conclusion of Ch XXXV, p 403

facts are dealt with below, under their respective headings, together with the findings of certain other workers

1. Origin of Seed Lymph

"Seed lymph" is a term applied in vaccine institutes for the inoculum employed for inoculating calves for preparation of vaccine pulp. The source of lymph used in different institutes varies somewhat, and it has been stated that there is no evidence to show that any difference can be detected in the quality of the lymph prepared from strains of virus originally derived from cases of smallpox or cowpox. However, from time to time the question has been raised of the protective value against virulent variola of vaccine lymph prepared either in distant countries, or from a nonlocal strain of vaccinia It has been felt that to obtain maximum protection against variola, a locally adapted vaccinia strain should be used. There is, however, no convincing evidence to support these views. For example, Horgan and Haseeb (1945) in the Sudan have used a strain of vaccinia deriving from a case of alastrim in London They found that this strain gave excellent protection against variola major. Pandit and Rao (1940) in rabbit experiments found that a recently adapted strain of vaccinia was not a more efficient immunizing agent than a stock institute strain

2. Maintenance of Virulence of the Vaccine Strain by Skin Passage

It is an established fact that the virulence of vaccinia can be maintained over an indefinite period by repeated propagation in calves. The virus is liable to lose its virulence if continuously propagated in the calf alone, and periodical cutaneous passage through rabbits may be adopted in order to exalt its virulence.

Many workers have observed the decline in the immunizing power of vaccine lymph resulting from lengthy passage through the same vaccinifer, and Blaxall (1921), among others, drew attention to this point, likewise, Cunningham (1927) found that after vaccine lymph had been passaged through 6 calves in succession, there was an appreciable decline in the insertion success-rate when tested on children The effect of repeated calf passage on the immunizing power of vaccine lymph has been investigated by Clearkin (1928–9), and Clearkin and Skan (1931), who proved that lymph showed diminished activity in the monkey, whereas lymph which had been propagated alternately between calf and monkey retained its potency (see also Lentz and Gins, 1927).

Another method for exalting the virulence of calf lymph is to passage it through man.

3. Neurolapine and Testicular Lapine

[illegible]

cinated human beings with neurovirus administered subcutaneously without demonstrable ill effects (see also Gonzalez, 1926). It would seem that the objections to the use of neurovirus for human vaccination are more theoretical than practical

TECHNICAL METHODS IN THE PREPARATION OF CALF LYMPH

The methods used in different institutes vary, and full descriptions of some of these are to be found in the publications of Blaxall (1930), Dalal (1930), Stuart and Krikorian (1930), King *et al* (1933), the *Report of the Committee on Vaccination* (1928) and Ducor (1947) We shall give a general account of the preparation of vaccine lymph as carried out in the majority of institutes at the present time

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addition of 2 grains of sodium bicarbonate to a mixture of 79 c.c. of glycerol and 100 c c. of distilled water, to which 0.1 per cent. of oil of cloves is added (Dalal, 1930). The necessity for using a diluent possessing an alkaline reaction, already known for many years, has been stressed by Godinho and von Klobusitzky (1934 *a, b, c*). The pulp is next passed through 2 triturating machines until reduced to a homogeneous consistency, so that the final product is clear in color and uniform in appearance if a loopful is examined with the naked eye against a bright light.

The Bacterial Content of Lymph

According to the *Report of the Committee on Vaccination* (1928), the bacterial content of lymph at glycerolation averages about 4,000 organisms per milligram, consisting mainly of staphylococci, bacteria of the *B mesentericus* and *B. subtilis* group, molds, sarcinae and yeasts (see also Belenky and Popova, 1929 *a, b, c, d*, 1930). These organisms are usually destroyed by the prolonged action of glycerol and oil of cloves during storage at − 11° C, so that after from 3 months to a year the number of bacteria falls to about 5 organisms per milligram, when the lymph may be fit for human use (see p. 378 for details regarding the bacteriological examination of lymph, and p. 379 for tests of potency) Large stocks are held in reserve and are issued in rotation according to demands, the usual age at issue being approximately 2 years from the date of collection. Subject to having passed the requisite tests for sterility and potency, the lymph is supplied for use and is put up in capillary tubes which contain about 0 02 c c., enough for a single inoculation

The comparative resistance of various pathogenic bacteria to the prolonged action of glycerol has been studied experimentally by Godinho (1934 *b*), who observed that if *Br. abortus, Br. melitensis, B. welchii, B. tetani*, staphylococci, *B. tuberculosis*, and gram-negative coliform bacteria were deliberately added to lymph and stored at + 10° C for 1 year, the 3 latter organisms were killed, while the others survived. Menon (1941) confirmed that storage in glycerinated lymph deprived staphylococci of toxicity and power of producing coagulase.

In the case of routine lymph production, the destruction of contaminant bacteria can be accelerated by placing the lymph at + 15° C for 4 to 7 days or even up to 14 days, depending on the retention of potency, or alternatively, by the action of weak antiseptics such as 0 1 per cent. oil of cloves, or 1 0 per cent. ether or chloroform (Dalal, 1930, Greaves, 1938). Numerous methods have been tried for reducing the bacterial content of calf lymph without diminishing its vaccinating properties. Gins (1921), Hackenthal (1930), and Lehmann (1937 *a*) have used carbolic acid as an agent for the destruction of contaminant bacteria in lymph, Gins employing the following method To the raw pulp he added 5 times its own weight of 1 per cent carbolic acid solution, this mixture was thoroughly triturated in a mortar, shaken in a receptacle for 4 hours, centrifuged at 3,000 r.p m. for 30 minutes, the supernatant fluid discarded, and an equal volume of sterile saline added. The last procedure was performed 3 times, in order to free the deposit of phenol, and after the final washing the water containing glycerol (to which agar had been added) was mixed with the deposit, to make up 5 times the weight of the original raw material Martin (1938) recommended the use of Gins's method

Butler (1938) has drawn attention to the value of phenol as an agent for preventing the accidental contamination of lymph He found that lymph, made up in 50 per cent glycerol containing 0 5 per cent phenol, when exposed to − 10° C, + 5° C, and + 15° C for 1 year, retained its potency better than lymph made up after the usual fashion in 50 per cent. glycerol containing 0.1 per cent. clove oil Kaiser (1937 *b*) was able to obtain calf lymph free from bacteria by grinding up the pulp in 0 3 per cent "zephirol," allowing it to stand for 10 hours, centrifuging the suspension, removing the supernatant fluid, and desiccating the deposit. X-rays have been employed by Levin (1935), who claims to have freed vaccine lymph from staphylococci and molds by exposure to the rays, without destruction of the

Seed lymph. This constitutes the stock virus used for regular propagation through calves, it is occasionally passaged through the skin of rabbits in order to maintain its virulence. Only the best and most typical vesicles are selected for seed lymph production, and rabbits are inoculated as follows:

Six to 8 weeks old white-skinned animals are shaved, the skin lightly scarified, vaccinia virus applied with a spatula, and when vesiculation has occurred (48 to 78 hours later), the animals are killed, the skin removed, stretched on a board, and the lesions scraped off with a Volkmann spoon. Material from several rabbits is pooled, weighed, *6 times as much 50 per cent. glycerol in distilled water added, and the* emulsion placed at $-11°$ C in the refrigerator. Such lymph is usually stored for a year or longer, after which it is examined bacteriologically, and if found to be free from pathogenic organisms and of moderately low bacterial content, together with high potency for the dermis of the rabbit, it is diluted 1/3 in 50 per cent. glycerol and used for inoculation of calves Seed lymph derived from calves is likewise treated (see Blaxall, 1930).

Calves. Only the healthiest beasts should be employed for vaccine production Each animal should be carefully selected by veterinary inspection during a period of observation in quarantine which, for lymph institutes in England and Wales, must extend for at least 5 days (Therapeutic Substances Act, 1931), after the vaccine has been collected the animal should be examined postmortem to insure freedom from *any diseased condition, especially tuberculosis Beasts varying from 4 to 6* months can be used.

Preparation of calf. The animal is thoroughly scrubbed with soapy water and antiseptic; the hair over the abdomen and inner side of the thighs is shaved with a razor, care being exercised to avoid cuts or abrasions on the skin; the denuded area is washed in water, dried with a sterile cloth, and 20 per cent. glycerol solution may be rubbed in to preserve the elasticity of the skin. After this treatment the animal is returned to its pen to be inoculated with vaccinia virus on the following day. Ducor (1947) recommends a technique of strict attention to cleanliness, and the use of "Roccal" solution on the skin of the calf; this results in the lymph having a very low final bacterial count.

Method of inoculation. The beast is strapped to a tilting table, is turned upside down, and the shaved area is very thoroughly washed by rubbing the skin for half an hour with soap and water, without the addition of an antiseptic; it is then dried with a sterile cloth, and inoculated with vaccinia virus. This is carried out by dipping the blade of a scalpel in a suspension of stock vaccinia seed lymph, usually diluted in 50 per cent glycerol and [illegible] of about 1 in 24, and then making a series of [illegible] ½ inch apart, these should not be d[illegible] *of the scalpel may then be drawn* [illegible] lymph into the abraded surface, and after the material has dried the animal is returned to its stall For the best results, the lymph should be inoculated in that area of the skin which lies below the malpighian layer and above the rete mucosum Vesicles usually form on the 4th or 5th day, and as these are liable to become dried up in warm climates, Dalal (1930) recommends the application of glycerol at intervals of 48, 72, and 96 hours after vaccination in order to prevent desiccation On the 4th day after vaccination, the rectal temperature rises

Collection of pulp Five days after vaccination the calf is placed on the operating [illegible] skin thoroughly washed with soap and [illegible] Each line of vesicles is then scraped off [illegible] cised in order to avoid, as much as possible, the presence of blood The pulp is placed in a sterile glass receptacle and its weight ascertained, this usually varies from 10 to 30 gm. per calf, and to it is added 4 times as much by weight 50 per cent glycerol in distilled water. The glycerol used has a specific gravity of 1.260, and is rendered faintly alkaline by the

plates and hemolysis in fresh rabbit's blood broth), the lymph is rejected. This is a very infrequent occurrence.

5. If any other pathogenic organism is found, the lymph is rejected.
6. If *B. coli* is isolated either aerobically or anaerobically, the lymph is re-examined (in 0.1 c.c. amounts in broth). If it persists after 3 such examinations at 3-monthly intervals, the lymph is rejected. If it disappears, the lymph may pass.
7. If streptococci other than *S. hemolyticus* are found, the lymph is kept and later reexamined in 0.1 c.c. amounts in serum broth. The lymph is not issued till it is free of such and, by experience, it is very seldom that it has to be rejected for persistence.
8. If hemolytic staphylococci are found, their characteristics are examined (sugars, action on gelatin, serum, and plasma). Because of dubiety as to what constitutes a bacteriological criterion of pathogenicity, it is considered advisable not to issue lymphs containing such in 2 milligrams of lymph, whatever the degree of hemolysis.

If the lymph is now considered satisfactory from the standpoint of bacterial purity and potency, it is put into capillary tubes. The lymph from sample tubes taken at the beginning, middle, and end of tubing is pooled and examined for the 3 criteria laid down by the Therapeutic Substances Regulations (Second Schedule, 1931), Part I (B), viz.

(*a*) Total count must be less than 5 organisms per milligram of lymph. Three separate milligrams are plated.
(*b*) Freedom from the presence of living hemolytic streptococci—smears on an agar plate are examined for streptococci.
(*c*) Freedom from the presence of living gas-producing anaerobic organisms in 0.1 c.c. of the lymph.

The properties of good lymph. These may be summarized as follows: a 0.1 c.c. sample should be free from hemolytic streptococci, hemolytic staphylococci, gram-positive sporing bacteria, and a colony count should yield a result of less than 5 per milligram of lymph.

The German Government regulations regarding the bacteriological examination of vaccine lymph are quoted by Wright (1935).

The Preservation of Vaccine Lymph

Akasawa (1936) stated that the addition of a buffered saturated solution of saccharose to glycerolated lymph enhanced its thermostability. Glue has also been employed as a diluent by Yaoi and Akasawa (1938).

Buddingh (1938*a*) recommended rabbit or ox serum as a protective diluent for calf lymph and also tried, without success, mucin, gum acacia, and egg yolk; suspensions of vaccinia virus purified by adsorption on kaolin were used for vaccination by Yaoi (1936*c*).

Certain workers have achieved a fair measure of success using dried lymph, and it may maintain potency for over a year if dried *in vacuo* from the frozen state; once reconstituted it must be used quickly (League of Nations, 1934; Boulnois, 1936, 1937; Kaiser, 1938; Morosov *et al.*, 1943).

Rabbit Tests for Potency of Lymphs

Under the Therapeutic Substances Act (Schedule 2, 1931) it is decreed that lymph to be used for human vaccination should possess a certain minimum of potency, and the tests advised are the four tests for potency recommended in 1927 by the Smallpox and Vaccination Commission of the League of Nations (see *Report of the Committee on Vaccination*, 1928).

1. *Gins's method.* In this method, a 1:1,000 dilution of lymph should produce

virus. Sonic vibrations were also tried for the purpose by *Yaoi and Nakahara* (1934), but with negative results

Penicillin added to lymph reduces the number of contaminant bacteria (Diaz Romero, 1945, Fasquelle, 1947; Morin and Turcotte, 1947).

Bacteriological Tests for Purity of Calf Lymph

The late Lt.-Colonel W. D. H. Stevenson, in a personal communication to us, recommended the following scheme for the bacteriological examination of lymph Repeated tests are necessary to obviate the risks attached to random sampling of a thick grumous material such as vaccine lymph The scheme is only intended for lymphs not immediately required Immediately after the lymph is prepared, counts for total number of organisms are made by pouring agar plates, and smears of lymph are made on agar plates for the detection of streptococci Then 0 1 c.c lymph is inoculated into Robertson's bullock heart medium under vaseline seal, the tube is heated to 65° C. for 30 minutes to kill nonsporing organisms, and thereafter incubated for 5 days at 37° C. The culture is then examined microscopically, and 0.5 c.c. injected intramuscularly into a mouse with 0.004 gm calcium chloride Also a large drop or large loopful of lymph (unheated) is inoculated into Fildes's blood digest broth, incubated, and from the growth the condensation water of a Fildes's blood digest agar slope is inoculated, this tube, after anaerobic incubation, is examined for the characteristic growth of *Cl. tetani*. After the tests are made, the bulk of the lymph is put into large containers which are not removed from the cold room (approximately − 10° C.) till towards the end of purification. The remainder is put into a small sample container which is brought up, as necessary, for examination. The contents of this small sample tube should be examined for the total counts and the characteristics of the organisms every 3 months When the number of organisms, which may vary from 1,000 to 10,000 per milligram, has fallen in 3 counts to 5 or less per milligram, all the contents of the large containers are examined, and will usually be found to have a considerably higher bacterial content owing to their steady storage at − 10° C If the count is high and the lymph is urgently required, it is incubated from 4 days to 1 week at + 15° C. and reexamined If necessary, a further period of 1 week is given at + 15° C When the contents of the large containers have approximately a bacterial content of 30 organisms per milligram, the lymph from each container is examined in detail. (*a*) Smears are made on agar and blood agar plates to detect streptococci or hemolytic staphylococci (*b*) 0 1 c c from each container is inoculated into Robertson's bullock heart medium The tubes are cooled below 37° C after a preliminary boiling before inoculation, have no vaseline seal but have wide-mouthed Durham tubes, and are incubated for 5 days in an anaerobic jar After 5 days the contents are examined microscopically, and 0 5 c c from each is inoculated with calcium chloride intramuscularly in mice Throughout, when the counts on agar plates are 10 or less colonies per plate, each colony shou[illegible] and, in any case, if streptococci, *B coli* etc, are [illegible] n detail It is recommended that the procedure [illegible] vents should be.

1 If at any stage a gas-producing anaerobe (if gas is not produced solely by the *B coli* group) is found, the lymph is rejected
2 If bacteria possessing terminal spores are found at any stage (the great majority produce gas in the anaerobic tubes in minute quantity) the lymph is rejected This occurs very infrequently
3 If a mouse dies and death is obviously due, or is proved on repeated examination to be due, to the injection of culture, the lymph is rejected. This very seldom occurs.
4 If *Streptococcus hemolyticus* is found (giving *β* hemolysis in blood agar

such tests, "strongly recommended that the medical directors of the vaccine institutes should whenever possible be given an opportunity of themselves undertaking human vaccinations, or at any rate, of satisfying themselves of the success of human vaccinations by personal observation" This is essential in centers where vaccination is not compulsory, and those engaged in the preparation of lymph have to rely on voluntary reports of doctors.

The term "insertion success rate" is applied to the percentage of insertions showing typical primary vaccinial takes when the lymph is inoculated in fully susceptible persons. It should be as near 100 per cent. as possible.

METHODS OF HUMAN VACCINATION

1. Application by Dermal Scarification

Present methods have evolved from the pioneer work of Dimsdale (1767), Bryce (1809), Seaton (1868), and others In Britain it has been recommended in the past that 4 separate insertions should be made, but the effects of this are too severe for general application. The Committee on Vaccination (1928) recommended that. (1) trial be made of vaccination or revaccination in one insertion with a minimum of trauma, and that crosshatching and multiple scarification be deprecated, (2) vaccination in multiple insertions be available for those requiring maximum protection The British Vaccination Order (1930) stated that vaccination should be performed ordinarily by a single linear incision or scratch not more than ¼ inch long, merely through the epidermis, but that under special circumstances up to 4 well-spaced insertions might be used

The skin at the selected site, preferably the left arm over the deltoid insertion, is cleansed with soap and water, and finally with alcohol, ether, or acetone. Some workers omit the chemicals, and in any event the arm must be absolutely dry before commencing to vaccinate. The contents of the capillary tube of lymph are expelled by a rubber teat onto one or more areas of the skin The contents must on no account be blown out, and virulent infection has been traced to this practice A heat-sterilized lancet is then used to make the selected number of scarifications through the lymph. They are usually about ¼ to ⅜ inch long, parallel and well spaced. The papillary layer of the true skin should be entered, and only slight bleeding caused. The lymph may be rubbed in with the lancet, and must be allowed to dry.

A sterile gauze dressing is often applied, but it is a better practice to use none until the lesion vesiculates

Vaccinations should be inspected, at least at the following times after the operation 48 hours, 7 days, 14 days.

2. Application by Multiple Pressure

This method has been particularly recommended by American workers (Leake, 1927, 1930, 1946, Grubbs, 1923), but outside North America has only been used by a few (Dudley and May, 1932, Parish, 1944, Mole, 1947). It is said to give an increase in the number of significant reactions on revaccination A drop of lymph is applied to the skin, and a Hagedorn needle (held parallel to the skin) moved rapidly up and down The trauma is caused by the side of the point of the needle. From 10–30 pressures are made according to circumstances, over an area of skin about ⅛ inch in diameter.

3. Intradermal and Subcutaneous Injection

Many workers have recommended that vaccine lymph, usually diluted, be injected intradermally or subcutaneously (Bussel and Stankiewicz, 1926, Toomey and Hauver, 1928, Kaiser and Hassmann, 1937, Muliner, 1937, Poch, 1938). The benefits claimed for these routes include milder reactions, absence of an "open" ulcer-

vaccinial keratitis in a guinea-pig 72 hours after inoculation of the eye. For this test the ground squirrel has been substituted for the guinea-pig by Goschanskaja and Stschastny (1933).

2. *Sobernheim's method.* Four patches are denuded of hair in a rabbit, and on each patch 3 linear incisions are made 4 cm. long and inoculated with different dilutions of lymph, from 1/1,000 to 1/10,000. The 1/1,000 dilution should show a specific reaction after 3 days.

3. *Gorth's method.* A quantity of 0.1 c.c. of a dilution of lymph varying from 1/10 to 1/100,000 is inoculated intradermally in the shaved skin of a rabbit, the lowest dilution should produce an area of local inflammation and redness in 24 hours' time, and the highest in 4 to 5 days. The size of each area of redness and infiltration can be measured with callipers, and by comparing the relative sizes of different areas it is possible to assess the potency of individual preparations of lymph. The standard recommended by the Commission was an area of redness and palpable swelling following inoculation of 0.1 c.c. of a 1/1,000 dilution of lymph in 72 hours.

4 *a. The Calmette-Guérin method.* In this method 1 c.cm. of vaccine lymph diluted 1/1,000, rubbed over the shaved back of a rabbit, should produce an eruption of 3 to 4 vesicles per sq cm. in 5 days.

4 *b. Blaxall's (1930) modification of Calmette's method.* The back of a rabbit is shaved, divided into 4 areas, and each of these is inoculated with 0 2 c c. of a 1/1,000 dilution of 4 different batches of lymph. Thus, with a lymph diluted to 4 times its original volume, the actual amount of vaccine pulp applied to each area is 1/250,000 gm A confluent eruption of vesicles should form on the 5th day after inoculation, if the vaccine is of adequate potency. In performing the test, care should be taken to scarify the skin lightly before applying the vaccine.

Stevenson (1938) personally informed us that 2 rabbits should be employed for each test, since individual animals vary greatly in their relative susceptibility to vaccinia. The maximum potency of the lymph also supplies valuable information and, therefore, a 1/10,000 dilution of lymph should be inoculated at the same time as the 1/1,000 test is conducted Further data on the examination of calf lymph for potency appear in the extensive report of Stevenson and Butler (1939) on the cultivation of vaccinia virus on the chorio-allantoic membrane of the chicken embryo (see also Force and Leake, 1927).

Issue

After a batch of lymph has passed the requisite tests for bacteriological purity and pathogenicity for the rabbit, it is sealed in capillary tubes and issued for human use Capillary tubes are filled with vaccine lymph by using the Enterican machine.[1] The method is as follows Several tubes, sealed at one end, are clamped in a holder and lowered into a vacuum chamber containing at its foot a quantity of glycerolated calf lymph. As soon as the open ends of the tubing are immersed in the fluid, air is released into the chamber, the lymph flows upwards into the tubes which are thereafter removed, sealed with aseptic precautions, packed and dispatched for use (see also Kaiser, 1937 *a*)

Human Tests for Potency of Lymph

Although lymph which has passed any one of the 4 rabbit tests for potency is fit for human vaccination, it is customary in most institutes to issue a limited quantity from each batch of lymph so that preliminary trials may be conducted on human beings prior to its use among the general population The League of Nations Sub-Commission on Vaccination, referring to the advisability of performing

[1] See Messrs Baird & Tatlock's, Ltd, Catalogue No BP860, p. 1954, Section XXIV, 7th edition.

monkeys and rabbits. Neutralizing antibodies develop to the same titer after inoculation of egg or calf virus and persist for over a year (Buddingh, 1943).

It appears that this method of vaccination may be of the greatest value, and may eventually supplant the older-established methods At the moment, however, there is a natural hesitation to abandon the time-honored method of scarification with lymph, which has been proved effective, beyond any doubt, in controlling smallpox.

7. Vaccination with Tissue Culture Virus

Virus cultured *in vitro* has been used for jennerian vaccination of man by various authors with satisfactory results (Rivers and Ward, 1931, 1933 *c*, 1935, Rivers, 1933, Coffey, 1934, Gallardo, 1934 *a*, Togounova and Baidakova, 1934, Bucher, 1935, Togounova *et al*, 1935, Barg and Borodaj, 1936, Plotz and Martin, 1936, Gallardo and Sanz, 1937, Plotz, 1939, Ellis and Boynton, 1939, Donnally *et al*, 1940).

Elsewhere we describe the technical details involved in the preparation of virus suitable for human inoculation (see Ch. XII). After the intradermal injection of 0 1 c c. of glycerolated culture virus, a small red papule develops in 4 to 6 days, this becomes larger and the zone of induration and erythema measures 2 to 3 cm in diameter (Rivers and Ward, 1935). *Four to 6 days after the beginning of the* reaction, there is noted a secondary less intense area of erythema, which lasts for a few days. Induration lessens progressively and is usually absent 3 weeks from the beginning of the rash Rivers and Ward found that primary vaccination with culture virus produced immunity to revaccination with calf lymph However, it has been found that the state of immunity induced by cultured virus may be of shorter duration than that induced by ordinary lymph (Donnally, 1939)

There are important advantages in the use of tissue culture virus over calf lymph, for it can be inoculated intradermally or subcutaneously, and it can be obtained bacteria-free Further, it is more economical, Herzberg (1932), for instance, found that 1 chick embryo medium (50 c c. bulk) yielded as much vaccine as 8 to 10 calves Culture virus can also be stored quite readily, and virulence maintained

8. Vaccination with Elementary Body Suspensions

Henderson and McClean (1939) inoculated an elementary body suspension intracutaneously, by introducing the needle for 8 mm and producing a wheal at the point of the needle, away from the site of insertion They found there was a definite correlation between the appearance of a typical vesicle following the primary vaccination, and the subsequent development of resistance to revaccination

Gastinel and Fasquelle (1941 *c*) have also vaccinated with EB suspensions

9. Vaccination by Other Methods

Vaccinia may be applied to the nasal mucosa (Gyure, 1939) Virus treated with supersonic waves has been used (Kasahara and Ogata, 1939) Vaccinia grown in culture may be mixed with yellow fever vaccine and applied by scratch (Peltier *et al*, 1939, 1940). Vaccinia and TAB may also be combined and injected subcutaneously (Yaoi, 1939, 1940, Yaoi, Hirose, and Sudzuki, 1939)

It has been recommended that in children at the time of vaccination, diphtheria prophylactic should also be given (Stern, 1935, Iavorschi, 1936).

THE APPEARANCE OF THE PRIMARY VACCINIAL LESION

Three days after vaccination, a small red elevated papule appears at the site On the 5th day, fluid accumulates, and the center of the lesion becomes umbilicated This is the vesicular stage The vesicle extends and on the 8th day appears

ated area, minimal scarring, more accurate assessment of dosage, and smaller consumption of lymph

Subcutaneous injection has been extensively used by Yaoi with bacteria-free lymph (1934, 1935 *b*, 1936 *a*, 1938 *a*, *b*, 1941). Other workers have recommended this route (Gherardini *et al*, 1939, Kaiser, 1939). After this route of injection, an area of infiltration develops between the 7th and 12th days, and this may be accompanied by fever.

Intradermal injection is economical in lymph, as a single tube can be used for about 20 persons, this method has been recommended by a number of workers (Roberts, 1932, Torello-Cendra, 1936, Pierce, 1937, McEwan, 1940, Pottinger, 1940).

Pierce and Willoughby (1943) insert a triangular surgical needle intradermally through a drop of lymph, the needle passing in for 1/16 inch parallel to the skin surface.

These 2 routes of inoculation may be combined (Leiner and Kundratitz, 1921, Simkó, 1925, Rosenbusch, 1936).

4. Vaccination with Virus and Antiserum

Picardo (1931) injected intradermally a mixture of calf lymph and antivaccinial rabbit serum, and claims to have reduced the area of scarring as well as general *malaise*

5. Vaccination with Killed Vaccinia Virus

This has been tried by several workers, but the results as a whole have not been encouraging. Henseval (1919 *b*) found that vaccination with lymph heated to 60° C resulted in a certain amount of protection, but after heating to 70° C. the antigenic property of the lymph was lost Brokman, Bussel, and Mayzner (1930 *b*) also claimed to have vaccinated children successfully using heat-killed or formolized virus, and Bussel and Mayzner (1930) obtained similar results after subcutaneous *or intradermal injection of killed virus*

6. Vaccination with Egg Culture Virus

Vaccine growth in the fertile egg has the following advantages it can be obtained completely bacteria-free, it is relatively simple to prepare, it is cheap, and obviates the necessity for using calves, and this may be a great asset in *the tropics* (Bourguignon, 1936), finally, if at any time there is an extraordinary demand for vaccine, this can be met quickly by inoculating a sufficiency of eggs The originators and leading exponents of this technique have been Goodpasture, Buddingh, and their collaborators in America (Goodpasture and Buddingh, 1933, 1934, Good- [illegible] d in Great Britain (Steven- [illegible] Lehmann (1936 *b*, 1937 *b*, [illegible] in other authors have carried out human inoculation experiments with egg-cultured virus (Godinho, 1934 *a*, *Herzberg, 1935*, Taniguchi *et al*, *1935*, *Munsterer, 1936*, Perini, *1936*, Peragallo, 1936–7, Gallardo and Sanz, 1937, Lazarus *et al*, 1937, Ellis and Boynton, 1939, Gastinel and Fasquelle, 1941 *a*, *b*, Balozet, 1942, Buddingh, 1943, Nagler, 1944)

Elsewhere we describe the technical details of egg passage, and the preparation of vaccine suitable for human inoculation (see Ch XIII) With regard to the results following the use of such vaccine, we may cite certain *observations* For example, Goodpasture and Buddingh (1933) found that, compared to calf lymph, the reaction produced by egg virus was slightly milder, there was less induration and pustulation.

Later, Goodpasture *et al* (1935) claimed that the inoculation of human beings with egg-cultured virus gives as good results as calf lymph, further, *significantly* similar antigenic powers are exhibited by egg virus and calf virus when tested in

1. A reaction reaching its maximum diameter on the 8th–14th day is known as the primary vaccinial response, and occurs in the fully susceptible person (see above).

2. A reaction occurring in those possessing partial immunity, in which the areola reaches its maximum on the 3rd–7th day, is known as the accelerated or vaccinoid reaction.

3. In the immune person, the areola reaches its largest diameter between 8 hours and 3 days. This response is termed the "immune" or immediate.

Incidence of the Various Lesions

The observations of Broom (1947) are of great value, and are shown in Table 16 He performed 1364 vaccinations in adults, mostly revaccinations It will be noted that control reactions to heated lymph occurred in persons revaccinated at intervals of 0 to over 20 years after the last vaccination The relative number of primary takes rose with the lapse of time since the last vaccination, whereas the relative number of immune reactions fell with the passage of time. The proportion of accelerated reactions lay between 40 and 50 per cent. at all periods.

TABLE 16

RESULTS OF 1364 VACCINATIONS AND REVACCINATIONS IN ADULTS (BROOM, 1947)

Years since last Vaccination	*Primary*	*Accelerated*	*Immune*	*Nil*	*Total*	*Control Reactions (Heated Lymph)*
0–2	9	67	64	20	160	14
2–5	15	97	40	43	195	25
5–10	8	48	8	25	89	10
10–15	16	34	0	20	70	8
15–20	25	54	3	30	112	16
>20	213	284	4	100	601	37
Total	286	584	119	238	1227	110
Primary Vaccinations	135	2	0	0	137	—

Technical Considerations in Revaccination

In revaccinating, the lymph used should be of high potency, for lymphs giving satisfactory results in susceptible persons may give indifferent results in semi-immunes (Horgan and Haseeb, 1944 *b*).

Revaccination is usually performed by 2 or 3 insertions, and Horgan and Haseeb found that the advantage of 2 over 1 insertion was very marked.

It is preferable to carry out the insertions in different areas of the skin, for there is some evidence that a local immunity may develop, revaccination of one site may give an unsatisfactory response, but on another area a successful result may be achieved.

A "Successful" Revaccination

This term should be restricted to the primary vaccinial reaction or the accelerated reaction, i e, the appearance of a vesicle should be the criterion of success. These reactions raise the level of immunity, and a person who so reacts may be regarded as protected against smallpox.

The Significance of the Immediate or "Immune" Reaction

[illegible] + to ++++ by the New York Quaran- [illegible] n in the test insertion with an insertion of [illegible] vel of immunity, and it should not be regarded as a "successful" revaccination, failure to understand this point has resulted in a great deal of confusion. Numbers of cases of smallpox have been recorded in

as a firm white swollen lesion On the 9th day, pustulation occurs, and there is a surrounding areola measuring an inch or more. From the 11th day, the pustule undergoes drying, and within the next few days the lesion becomes converted into a scab which eventually falls off, to leave a cicatrix visible usually for life.

According to Regan (1944) the success of a vaccination is indicated by the appearance of a minute white papule with a pink areola appearing within 3–10 minutes of vaccination, and lasting for 20–30 minutes, a hand lens is required.

[illegible]

caused by the presence of fluid, this fluid contains elementary bodies Some intracytoplasmic (Guarnieri) bodies may be found in the cells around the lesion. The contents of the pustule consist of polymorphonuclear leukocytes, a few red cells, fibrin clot, and occasionally secondary invading organisms such as staphylococci, especially if the skin has been abraded through mechanical irritation or scratching The crust or dried scab is composed of desiccated serum and fibrin clot containing disintegrated cells, and the deeper the involvement of the cutis vera, the longer the scar lasts (see Turnbull and McIntosh, 1926). The changes in the epidermis and dermis are said to be much severer in human vaccinia than variola (Howard and Perkins, 1905–6).

Primary vaccination in infants Newborn infants may exhibit resistance to vaccination, this resistance has usually disappeared by the age of 3 months (Schossmann and Herzberg-Kremmer, 1932; Béclerè, 1936). Various views have been put forward to account for this insusceptibility, such as transmission of maternal immunity (see Bennett, 1936; Makgill, 1936), or a natural immunity (Lukacs and Moritz, 1935).

In adults. The general opinion is that adults tolerate primary vaccination less satisfactorily than children, and severe reactions may occur (see Holmgren and Lindstrom, 1937). When a primary vaccinial response occurs in an adult previously vaccinated in childhood the reaction is usually milder (see Gyllensward, 1934, Lindeman, 1934; Halldén, 1937).

USE OF CONTROLS IN REVACCINATION

In order to simplify the interpretation of the response on revaccination, and to distinguish between sensitivity and immunity, it is recommended that at the same time a separate insertion of heated lymph should be inoculated (see, e.g., Hooker, 1925, Broom, 1947, and Table 16).

THE RESULTS OF REVACCINATION

Following revaccination, if immunity induced by the first inoculation has completely waned, a primary vaccinial lesion may develop, and this has been described above. More usually, however, some type of modified response, i e., the immediate or the accelerated reaction, occurs Finally, the revaccination may "fail to take." The interpretation of some of these reactions presents considerable difficulty to vaccinators, and numerous workers have discussed their significance (e g, Leake, 1923, 1927, 1936, 1946, Hooker, 1925, Andervont and Rosenau, 1930, Brokman *et al*, 1930 *a*, McKinnon and Defries, 1931, Bull and Rankin, 1936, Doraisingham, 1940, International Sanitary Convention, 1944, War Office, 1944; Seal, 1945, Mitman, 1947). Particularly valuable discussions that we have consulted are those of Stevenson (1945) and Marsden (1946).

Appearance of the Lesions

Leake defines the reactions as follows, measuring the maximum diameter of the red areola.

sensitization can be effected by repeated injections of killed virus (Gastinel, Reilly, and Mortier, 1932).

SERUM ANTIBODIES IN REVACCINATION

Although there has been a number of studies carried out, there is insufficient evidence to show whether antibody responses can be correlated with the results of revaccination One can only s [illegible] "immune" reactions show the presence of [illegible] (Force *et al*, 1929; Blattner, Heys, and [illegible] glutination-inhibition technique (Ch. XXXI) suggest that a greater rise in antibody occurs after a successful vaccination or revaccination than when an "immune" reaction occurs.

CERTIFICATES OF VACCINATION

Certificates should preferably be given in the form recommended by the International Sanitary Convention (1944) which is reproduced in Fig. 30. It is unfortunate that various bodies fail to accept a certificate of "failure to take," for certain immune persons will not show any other reaction, even on repeated vaccination. It is wiser to adopt the policy of the British Army authorities and give a certificate of "insusceptibility to vaccinia," after 3 attempts to produce a reaction, using multiple insertions, have failed (War Office, 1944)

IMMUNITY TO VACCINIA AFTER VACCINATION

A successful vaccination usually protects against the development of primary vaccinia on revaccination for many years, estimated at from 10–20 or even longer, such previously vaccinated persons tend to develop a modified type of reaction on revaccination (Dudley and May, 1932, Deiring and Rosenau, 1934, Gyllenswärd, 1934, 1936, Reiter, 1935; Loy and Husband, 1938). However, immunity may be much shorter, and Stevenson (1945) quotes a number of cases where revaccination has been performed successfully comparatively recently after the first vaccination There is no definite evidence that these "takes" after short intervals are commoner following vaccination by one insertion, than vaccination by multiple insertions.

The table published by Broom (1947, see above) gives valuable information regarding the likelihood of primary vaccinia developing at varying periods after vaccination

THE VALUE OF VACCINATION AS PROTECTION AGAINST SMALLPOX

The protective power of vaccination (or variolization) against infection with smallpox is one of the best established facts in preventive medicine. The subject can be discussed from 2 angles The ability of vaccination to lower the incidence of smallpox in the community in general, i.e., herd immunity, and the protective power of vaccination in the exposed individual

During and since the second world war, smallpox became a problem to service and other medical officers whose knowledge had hitherto been theoretical rather than practical. Many British and other writers displayed unfamiliarity with the lessons of the past, acquired, for example, at the beginning of this century when smallpox was a serious problem in the British Isles, and taught in particular by Ricketts. Certain authors have done valuable service in restating the true position in regard to the protective powers of vaccination, especially Stevenson (1944) and Marsden (1946)

Immunity in the Individual

No one will question the protective power of vaccination for the individual exposed to smallpox. This is so well attested that it is not necessary to quote extensive references. A striking example is afforded by Marsden's experience in Lon-

soldiers and others who have a record in their papers of an "immune" reaction—sometimes recorded as a "successful" vaccination (see, e.g., Cottrell and Knight, 1939, Illingworth and Oliver, 1944, Boeck, 1946; Marsden, 1946). [In fact, entries, as in pay books, that vaccination was performed on such and such a day are no indication at all that the person concerned is resistant to smallpox (see, e.g, Coleman, 1944; Black, 1945).]

The "immune" reaction is badly named—the term "immediate" is preferable—because this type of lesion may occur under the following conditions

1. It may be due to trauma only.
2. A similar lesion occurs in the early days of an accelerated reaction.
3. It may be due to weakly potent lymph.
4. It may be specific, but only due to sensitization to vaccinial protein acquired by a previous vaccination, and be equally well elicited by killed virus (see below).
5. It may be specific and indicate immunity to live virus, inoculation of killed virus giving a negative result.

Brom (1947) questions the value of the "immune" reaction, for persons susceptible to living vaccinia virus may react to heated virus in a manner closely simulating the immune reaction. Further, persons may be immune to live vaccinia as shown by failure to "take" on repeated revaccination, yet they do not show the reaction of immunity.

It is evident, therefore, that the reaction of immunity can be recognized with certainty only if there is a transient papule surrounded by an areola at the site of insertion, and there is no reaction with heated lymph.

The position is admirably summarized by Marsden (1946) who states "while it is probably true that an immediate reaction is the normal response to vaccination of the completely immune individual, the evidence that this reaction can occur in a subject who is susceptible to vaccinia or to smallpox appears to be overwhelming."

Significance of "Failure to Take"

It cannot be emphasized too strongly that the commonest cause for a failure to "take" on revaccination (or vaccination) is technical Either the lymph has become impotent from exposure to atmospheric temperature, or the scarification has been too superficial, or the antiseptic used to cleanse the arm has not dried, and has inactivated the virus.

When a revaccination does not "take," the person must be revaccinated on 2 or 3 occasions on different areas of skin. In practically every case some type of positive reaction will occur This has been emphasized by recent workers (D'Arcy, Moore, and Whetter, 1943, Marsden, 1946, Mole, 1947). Now and again one meets with the person who, though carefully observed, fails to show any response It is best to issue these persons with a certificate of "insusceptibility to vaccinia" Some of these persons may be immune to variola, but it is most unwise to regard this as established. There is a well known example in the literature of a soldier vaccinated 10 times in one year, failing to "take" on each occasion, yet dying of smallpox (Easton, 1945) Other workers have described cases of smallpox shortly after a "failure to take" (see, e g , Beaumont, 1946)

ALLERGY IN REVACCINATION

Persons who have been previously vaccinated may develop a sensitivity to reinoculation of virus, and this can be demonstrated with heated antigen (von Pirquet, 1907, 1911, Hooker, 1925, 1929, Leake, 1936) It can also be elicited with killed and washed suspensions of EB's (Craigie and Wishart, 1933) The reaction comes on within 3 days, and is identical in appearance with the immediate reaction No such reaction occurs in persons being vaccinated for the first time De-

vaccinial vesicle may induce an immunity to smallpox lasting for several years, usually about 10, the duration is very variable, and in certain cases may be quite short, about 2 years. It is probable that immunity can be broken down by an overwhelming dose of the infecting agent. Stevenson (1944) quotes a number of published cases of smallpox occurring at short intervals after successful vaccination, *even under one year. Modern practice is to advise "little and often" as the guiding* rule for persons likely to be heavily exposed, such as health and port officers, medical men, and nurses, and to revaccinate every 6–12 months. We may regard 4–5 years as the average duration of resistance, but this may often extend to a longer interval.

Smallpox in the vaccinated. The person who has acquired resistance to smallpox by vaccination usually suffers from a modified type of illness, ranging from the mild "contact fever" of the highly immune to the atypical case with rash. This question is fully discussed on page 294. It must be emphasized that the protective power of vaccination "wears off" in a definite order, and that protection against the early toxemic phase is lost before the rash-inhibiting property. This characteristic points to the wisdom of frequent revaccination of highly exposed persons.

Influence of number of insertions on protection. There has been much discussion regarding whether greater protection is afforded by several insertions than a single one. The Committee on Vaccination (1928) concluded that the greater the number of vaccinial scars, the more the protection against death from smallpox Thus from 1901–1904 the mortality among those showing 4 or more scars was 5.44 per cent, but 17.94 per cent. in those with only a single scar. The death rate was lowest (6.95 per cent.) in those who had a minimum vaccinated area of half a square inch, and highest (16.93 per cent.) in those where the area was less than one-third of a square inch They concluded that the number of inoculations had more significance in regard to protection against death than the combined areas of the scars. Lehmann (1936*a*), on the contrary, criticized these conclusions.

Stevenson (1944) points out that apparent failures to protect have followed vaccination by one as well as multiple insertions. It is widely accepted, nevertheless, that multiple insertions do confer greater protection

Marsden (1946) concludes that the state of our knowledge regarding the protective value of single mark vaccination remains where it was—there is yet no conclusive evidence. He points out, however, that recent practice has been to recom*mend 3 insertions in the face of an epidemic. Certainly this practice is to be* commended.

Influence of potency of lymph. Although it has been suggested that the potency of the lymph determines the degree and duration of protection, Stevenson (1944)

per cent. or 100 per cent. "takes" in a large number of people.

Immunity in the "Herd"

It is the general experience that vaccination of disciplined bodies such as troops lowers very markedly the incidence of smallpox when the men come into contact with the disease, e g, in the civilian population Oft-quoted evidence is that of the Prussian Army in the Franco-Prussian War (see Stevenson, 1944) However, it is recognized that even in such bodies of troops, smallpox will occur, and complete freedom cannot be expected, especially if the disease is prevalent in the civilian population

There is evidence that vaccination of civilians in endemic areas also tends to lessen the general incidence of the disease. A few examples may be quoted For example, owing to the geographical position of Palestine, the disease was at one

INTERNATIONAL CERTIFICATE OF VACCINATION AGAINST SMALLPOX

THIS IS TO CERTIFY THAT . . . .

(Age . . .. Sex . .) whose signature appears below has this day been vaccinated by me against smallpox.

Origin and Batch No. of vaccine ....

Official Stamp	Signature of Vaccinator . ...
	Official Position .. . .
	Place .... Date ....

Signature of person vaccinated . . ..

Home Address ....

Important Note: In the case of primary vaccination the person vaccinated should be warned to report to a medical practitioner between the 8th and 14th day, in order that the result of the vaccination may be recorded on this certificate. In the case of revaccination the person should report within 48 hours for first inspection in order that any immune reaction which has developed may be recorded.

THIS IS TO CERTIFY THAT the above vaccination was inspected by me on the date(s) and with the result(s) shown hereunder:

Date of Inspection	*Result*
....	....
....	....
....	....

Official Stamp	Signature of Doctor . ...
	Official Position . ...
	Place .... Date ....

Use one or other of the following terms in stating the result, viz: "Reaction of immunity," "Accelerated reaction (vaccinoid)," "Typical primary vaccinia." A certificate of "No reaction" will not be accepted.

Signature of person vaccinated ....

(This certificate is not valid for more than 3 *years from date of issue).*

FIG 30 International certificate of vaccination against smallpox

don (1936) where from 1928–1934, 14,000 patients were treated in the London smallpox hospitals without a single case of the disease among the staff protected by vaccination

Time of onset It is generally agreed that by the time the vaccinial vesicle has formed, the vaccinated person is resistant to smallpox Vaccination after exposure to smallpox cannot be relied upon to prevent the disease, although it may modify it (see, e g, Macgregor, 1942, Clark, 1944), especially is vaccination likely to fail to protect if the vaccinial lesion takes longer to mature than usual (see Marsden, 1946)

Duration Although there is every reason to believe that the development of a

vaccinial vesicle may induce an immunity to smallpox lasting for several years, usually about 10, the duration is very variable, and in certain cases may be quite short, about 2 years It is probable that immunity can be broken down by an overwhelming dose of the infecting agent. Stevenson (1944) quotes a number of published cases of smallpox occurring at short intervals after successful vaccination, even under one year. Modern practice is to advise "little and often" as the guiding rule for persons likely to be heavily exposed, such as health and port officers, medical men, and nurses, and to revaccinate every 6–12 months We may regard 4–5 years as the average duration of resistance, but this may often extend to a longer interval.

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Influence of potency of lymph Although it has been suggested that the potency of the lymph determines the degree and duration of protection, Stevenson (1944) questioned whether the antigenicity of a lymph depends on potency, provided the latter is sufficient to insure a "take" That the lymph "takes" and produces a vesicle is all that matters for the individual He knows of no evidence that the protection in an individual vaccinated successfully is affected by whether the lymph gives 90 per cent or 100 per cent "takes" in a large number of people

Immunity in the "Herd"

It is the general experience that vaccination of disciplined bodies such as troops lowers very markedly the incidence of smallpox when the men come into contact with the disease, e g, in the civilian population Oft-quoted evidence is that of the Prussian Army in the Franco-Prussian War (see Stevenson, 1944) However, it is recognized that even in such bodies of troops, smallpox will occur, and complete freedom cannot be expected, especially if the disease is prevalent in the civilian population

There is evidence that vaccination of civilians in endemic areas also tends to lessen the general incidence of the disease. A few examples may be quoted For example, owing to the geographical position of Palestine, the disease was at one

time a serious problem, but by thorough vaccination control has been achieved (Balfour and Scott, 1924; Masterman, 1925; Stuart and Krikorian, 1930). In China, similar observations have been made (Uttley, 1938), and in Sweden (Halldén, 1937), and elsewhere (League of Nations, 1933). In India, Rogers (1945) notes the steady decline in the total smallpox deaths, and in average rates, coincident with a rise in the average numbers of vaccinations. At the period of the most rapid decline in smallpox incidence in British-administered territory, the death rates in states with little vaccination were up to 37 times as great as in neighboring fairly well-protected British-administered areas.

Useful evidence for the effect of vaccination in lowering the general incidence of smallpox comes from various American workers, many of whom draw attention to the different laws of the various states (e.g., Woodward, 1930, 1932, 1933, Feemster *et al*, 1932; Woodward and Feemster, 1933; Collins, 1936; Hedrich, 1936, Dauer, 1940, Hampton, 1943). The general experience is that where laws requiring vaccination for school attendance have been in force, smallpox has practically disappeared (Dauer). Hampton gives these figures: In states where vaccination is *essential for school attendance, the annual case rate of smallpox per 100,000* is 0.8, but in states where there are no definite regulations of value, the incidence is 11.1. In Canada important evidence comes from Groulx of Montreal (1940) who claims to have eradicated smallpox by measures directed to maintaining the number of vaccinated persons at the highest possible level.

More evidence comes from the results of mass vaccination in European cities. In recent experience, such campaigns were carried out in the Glasgow and Edinburgh outbreaks of 1942, when it appeared that the epidemic was "getting out of hand." The subsequent control of the epidemic appeared to be due to the raising of herd immunity following the introduction of mass vaccination. Not all, however, believe that the raising of herd resistance by this means is the cause of the cessation of the epidemic; they suggest that the epidemic is already virtually completed at the time of introduction of mass vaccination (see in particular, Millard, 1943 *a*, *b*, 1945 *a*, *b*).

IMMUNITY TO VACCINIA AFTER SMALLPOX

Immunity to vaccinia becomes complete a few days after the appearance of the focal rash, when vaccination no longer "takes." For example, in the London alastrim outbreak Marsden (1936) found that 34 per cent of 377 patients vaccinated on the first day of the focal rash developed a successful vaccination, but no vaccination was successful after the 6th day of the outcrop.

Although second attacks of smallpox are unusual, it is not infrequently possible to vaccinate successfully a person who has suffered from smallpox in the past (see, e.g., Dearing and Rosenau, 1934).

DISTRIBUTION OF VIRUS IN THE VACCINATED SUBJECT

Although the majority of vaccinated persons does not show any obvious signs or symptoms of generalized vaccinial infection, there is good reason to suspect that the virus is capable of entering the blood stream and causing a transient and generalized invasion of various organs. Thus, Herzberg-Kremmer and Herzberg (1930–1) found the virus in the blood in certain normal cases of vaccination between the 3rd and 10th days after inoculation. Virus was usually absent from the cerebrospinal fluid and the pharynx, but in certain cases, where the response to inoculation was excessive, it could be recovered from the cerebrospinal fluid, blood, and pharynx. The virus has been isolated from the tonsil (Gins *et al*, 1929) and nasal passages (*Goschanskaja et al*, 1932). Walthard (1926) states that he failed to isolate vaccinia virus from the cerebrospinal fluid of 12 patients 12 days after vaccination. Eckstein (1930) found no evidence of increased meningeal permeability, using the bromine test.

VACCINATION AND PREGNANCY

There is no evidence to suggest that vaccination during pregnancy interrupts the normal course of events. Furthermore, attention has been drawn to the fact that even if the fetus becomes infected, miscarriage does not always occur, and Lynch (1932) has quoted 47 cases in which children were born alive with pustular lesions on their skin (see also Marsden and Greenfield, 1934)

COMPLICATIONS OTHER THAN ENCEPHALITIS

Very rarely vaccination, and even less commonly revaccination, is followed by a complication

Nonspecific Vaccinial Rashes

A nonspecific rash may develop 6–14 days after vaccination. In most cases the rash is of the erythema multiforme type, morbilliform, or urticarial A causal factor may be the absorption of small traces of foreign serum from the lymph, or of toxic substances from the pustules Various workers have described rashes of this type (Rolleston, 1925, Chalke, 1931, 1941, Akiyama, 1937, McKenzie, 1943, Clark, 1944, Director, 1944) Bloch (1942) reported over 100 postvaccinial rashes out of half a million vaccinations performed in Glasgow in 1942 Most of these developed 7–11 days after primary vaccination in children but the limits were 2–49 days afterwards, most were erythemas, some were papular urticaria, and some were erythema multiforme.

Generalized Vaccinia

In a small proportion of cases, a generalized vaccinial eruption (from which the virus can be isolated) develops 6–14 days after vaccination (Drago, 1924; Gins, 1930*b*, de Vere Shortt, 1933, Dible and Gleave, 1934, Chaudhuri, 1935, Ellis, 1935, Ohta, 1935, Evans, 1937, Rolleston, 1937, Wollnitza, 1938, Eldahl, 1939, Regamey, 1940, Barber, 1942, Coombes and Behrman, 1943, Jubb, 1943, Clark, 1944, Director, 1944, Finn, 1944, Wollenman, 1944, Gropper, Lamanna, and Stewart, 1945; Belam, 1946).

In some cases of this sort, infection is probably transferred by auto-inoculation, with the fingers In true generalized vaccinia, the virus normally present in the blood of vaccinated persons localizes in the skin, the syndrome being analogous to generalized vaccinia in the rabbit Lesions of the skin may predispose to the localization of the virus It has been suggested that there is an association between allergy and postvaccinial manifestations (Davidson and Davis, 1943)

The serum of a recently vaccinated person has been used with success in treatment (Chesney and Jubb, 1944)

Accidental (Heterogenous) Vaccinia

Persons not themselves bearing a vaccinial lesion may be accidentally infected by contact with a case of vaccinia, and develop lesions on the skin 6–10 days later, the source of infection may be soiled bandages, towels, or bath water (de Vere Shortt, 1933, Clark, 1944).

Children suffering from eczematous skin eruptions are prone to develop vaccinial eruptions if they come in contact with a vaccinated person (Graves and Dowman, 1937, Albrigo, 1938, Snoek, 1938, Schmid, 1940, Brown, 1944, Petersilge and Toomey, 1944, Hershey and Smith, 1945) Burns are also liable to become infected with vaccinia (Nimpfer, 1936) The term eczema vaccinatum or heterogenous vaccinia may be applied to this condition Accidental infection of the nose has been reported (Ayo and Braley, 1942)

Postvaccinial Tetanus

This may be due to contamination of lymph with tetanus bacilli, but more commonly the infection is introduced by dirty earth-soiled fingers or dressings (see *Colò*, *1932*, Recchia, 1939, *Ambrosioni*, *1940*). *Felt* toe pads have given rise to tetanus after use as dressings for vaccination sites (Armstrong, 1925, *1929 b*, McVail, 1925).

Vaccinial Infections of the Eyes

The conjunctivae can become infected by auto-inoculation (Folk and Taube, 1933, Sezer, 1938, Atkinson and Scullard, 1940; Laval, 1940, Berkley, 1942, Klumzinger, 1944; Brav, 1945). The condition of keratitis postvaccinolosa is described (Sinaiko, 1931), also vaccinial diskiform keratitis (Perera, 1940).

Rarer Complications

The formation of *keloids* in the vaccination scar has rarely been reported (Louste and Rabut, 1932). *Glomerulonephritis* has been observed following revaccination (Herbut, 1944). Vaccination may cause an exacerbation in a latent tuberculous focus (Keers and Steen, 1943).

Vaccine lymph has been found contaminated with the virus of foot-and-mouth disease (Magnusson, 1930, van Heelsbergen, 1931), but there is no evidence that this is more than an extreme rarity (Gildemeister, 1931, Gildemeister and Helm, 1931–2).

THE SAFETY OF VACCINATION

It cannot be emphasized too strongly that all the complications mentioned above are so exceedingly rare in occurrence that they should never be regarded as arguments to be used against vaccination, since the safety of the procedure is absolutely unquestionable; Rhodes (1930), for example, mentions that he vaccinated 16,000 persons within a period of 4 months at the time of an epidemic, without a single case of ill effects being reported Likewise, Donnally and Nicholson (1934) successfully vaccinated 500 newborn infants against smallpox, and found the procedure to be perfectly safe, for it neither retarded the growth nor affected the nutrition of the child, and was usually unaccompanied by fever.

Statistical data indicating the total number of primary vaccinations and deaths from vaccinia at all ages and under one year old, in England and Wales from 1875 to 1925, have been summarized in the *Report of the Committee on Vaccination* (1928) These figures further emphasize the comparative rarity with which complications are encountered during the course of vaccination and reveal that in the period 1886 to 1891, during which 4,290,000 infants were vaccinated, there were 279 deaths at all ages, whereas in the years 1911 to 1925 when 5,500,000 were inoculated there were only 128 deaths at all ages

It has been said that severe vaccination reactions are commoner in the stout, and in women (Buchanan and Laidlaw, 1942).

THE WASSERMANN REACTION IN VACCINATION

Vaccination is one of the conditions that gives rise to a biological false positive WR (see, e g, Lynch, Boynton, and Kimball, 1941, Thomas and Garrity, 1941, Favorite, 1943, 1944, Rein and Elsberg, 1945).

CHAPTER XXXV

POSTVACCINAL[1] ENCEPHALITIS

FOR some years very considerable interest has been taken in an occasional sequel of the usually harmless procedure of vaccination. Such cases of postvaccinal encephalitis were probably first observed in 1905 and 1906 by Comby in France (see 1926), and later, in 1912 by Turnbull in London (see Turnbull and McIntosh, 1926). It was not, however, until 1922–3 that the disease assumed serious proportions. At this time, a number of children were stricken with a grave, often fatal, nervous illness some 10 or more days after a routine, apparently otherwise normal, vaccination.

Holland was particularly visited, and the complication had a considerably higher incidence there than in other countries Numerous cases occurred in Britain, and formed the subject of many papers and official reports Soon it came to be realized that the incidence of this condition was more widespread than at first supposed, [illegible] United States [illegible] It is related, [illegible] vaccination, and other conditions

With regard to literature, in Britain the disease was first reported officially by the Andrewes Committee, which met in 1925, and next by the Rolleston Committee, which was appointed on February 4, 1926. The findings of these committees were published in an official report in 1928 (*Report*, 1928). The Rolleston Committee published a further report in 1930 (*Report*, 1930). These reports are most comprehensive, and should be studied by all interested Certain other reports and reviews should also be read (Levaditi, Nicolau, and Sanchis-Bayarri, 1927, *League of Nations Report*, 1928, Armstrong, [illegible] Flexner, 1929 *a*, *b*, Matheson Committee, [illegible] ge, 1931, see also, 1932, Thomp- [illegible] Bogaert, 1937, Sindbjerg-Hansen, [illegible] some in the British outbreaks of [illegible]

CLINICAL FEATURES

Incubation period. The commonest incubation period is from 10 to 13 days, the day of maximum incidence being the 12th. Cases have developed, however, within the limits of 2 to 26 days after vaccination.

Onset and course The child is usually taken ill suddenly, the symptoms are obviously cerebral, such as headache, squints, and vomiting The temperature is raised, and the intellect dulled Drowsiness is present and increases to unconsciousness in some days Paralyses, usually of the spastic type, may occur In certain cases the picture is more meningitic, with neck rigidity, eye changes, convulsions, and other evidences of meningeal irritation Kernig's sign may be elicited, and also the *tâche cérébrale* The vaccination lesions usually look quite normal, although on rare occasions they have appeared unduly severe.

Although the British cases described in the *Reports* of 1928 and 1930 were more or less uniform in symptomatology, elsewhere more varied manifestations were noted Lucksch (1926), for example, described cases as falling into the following types cortical parenchymatous, resembling encephalitis lethargica, meningitic, and

[1] The term "postvaccinal" is the one most commonly used, and we here follow this practice Elsewhere in the book, however, we use "vaccinial" as the adjective derived from "vaccinia"

Postvaccinial Tetanus

This may be due to contamination of lymph with tetanus bacilli; but more commonly the infection is introduced by dirty earth-soiled fingers or dressings (see Colò, 1932, Recchia, 1939; Ambrosioni, 1940) Felt toe pads have given rise to tetanus after use as dressings for vaccination sites (Armstrong, 1925, 1929 *b*, McVail, 1925).

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tion (see McIntosh, 1928, McIntosh and Scarff, 1927–8, Perdrau, 1928, Turnbull, 1928). Demyelination occurs in the form of wide "sleeves" around vessels, chiefly veins These areas sometimes fuse, to form large plaques Although Perdrau pointed out that the demyelinated area had no relation to the actual distribution of the vessel, it is now believed by some that vascular lesions play a predominant rôle in the causation of the demyelinating diseases

Globus (1932) drew attention to a source of error in examining small areas of supposed demyelination. He found that if such areas were closely examined, the myelinated fibers were often seen to be intact, but pushed away from the vessel by infiltrating cells, thus giving a false appearance of demyelination

2. *Perivascular infiltration.* There are usually a few cells infiltrating the adventitial sheath, perhaps one or two rows, where this infiltration is slight, the commonest cells are plasmacytoids More characteristic, however, is the occurrence of an extra-adventitial infiltration, composed of microglial cells and some polymorphs, hypertrophied neuroglia may also be found in these lesions, diffusely, or in a ring at the periphery (Perdrau).

3 *Diffuse infiltration* In cases where the extra-adventitial infiltration is very marked, there may be a more widespread increase of cells throughout the parenchyma

4 *Vascular changes* Hyaline thrombi may be found occasionally in cases of postvaccinal encephalitis Putnam (1937) has recorded the presence of more definitely organized thrombi Small perivascular hemorrhages may occur, but are unusual. Swelling and proliferation of the vascular endothelial lining is also a feature.

5 *Nerve cells* The most characteristic feature is that the cells are usually well

post-

it this

6. *Subacute lesions* Subacute cases have been reported where scarring was present, there was also an intense cellular infiltration, and fat-granule cells were present in large numbers in the vessel sheaths (Turnbull and McIntosh, 1926, Perdrau, 1928, Turnbull, 1928).

Distribution of lesions Inflammatory lesions occur particularly in the cerebral cortex, midbrain (especially substantia nigra), basal portion of the pons, and in the upper part of the medulla In the cerebrum and cerebellum the white matter is more involved than the gray (Perdrau).

As regards the cord, the infiltration is maximal in the lumbar region, the white matter, especially in the lateral and anterolateral columns, shows widespread demyelination

Changes in Other Parts of the Body

Perdrau (1928) recorded a mild lymphocytic infiltration of the posterior root ganglia in one case, no lesions were found in the brachial plexus or intervertebral ganglia of the affected side of the body by Coyle and Hurst (1929), thus rendering it unlikely that virus had spread to the central nervous system by nerve pathways

Turnbull and McIntosh (1926) found that the pathological features of the vaccination scabs, in cases of postvaccinal encephalitis, were absolutely normal (see p 384), neither did they find any lesions in the regional glands, apart from the nonpurulent infiltration present in normal vaccinated persons

In Russell and Oddie's (1928) case there was congestion of the kidneys Microscopically, the glomeruli were swollen, the nuclei were increased in numbers, the epithelium of the tubules showed cloudy swelling, and there was a deposit of calcium salts.

poliomyelitic, with chief incidence in the cord. Glanzmann (1927) mentioned the following varieties:

1. Meningeal symptoms predominated in this type
2. Encephalitic symptoms occurred with paralyses.
3. *Encephalitis was associated with tetanic spasms (see also Mader, 1929, Hassin* and Geiger, 1930).
4. Encephalitic symptoms of somnolence and cranial nerve palsies were present
5. The Dutch type of encephalitis was often fatal, with somnolence and convulsions, but no paralyses or spasms
6. The manifestations were those of disseminated encephalomyelitis with meningeal symptoms

Kaiser and Zappert (1938) have described bulbar cases. Peake (1929) and Magé (1937) have recorded cases of transverse myelitis (see also Brockbank, 1931, Style and Fleming, 1943; McKenzie, 1943, Dixon, 1944). Jermulowicz (1930) described 2 cases that resembled poliomyelitis (see also Perritt and Carrell, 1930).

Sequelae (which are uncommon) usually take the form of epilepsy and mental changes (see *Report*, 1928, Armstrong, 1932; Kaiser and Zappert, 1937).

CLINICAL PATHOLOGY

The cell count in the cerebrospinal fluid may be increased up to 100 to 150 mononuclear leukocytes per c mm; protein may also be increased (see *Report, 1928, Roper, 1933, Kaiser and Zappert, 1938). The white cells of the blood may* be increased, to 10,000 or even 18,000 per c.mm.

It is as well to carry out blood culture in all suspected cases, as it has been shown that septicemia may closely simulate postvaccinal encephalitis (Grey and Whittaker, 1930, Priest, 1930)

MORTALITY

The mortality figures have, of course, varied from time to time. The case mortality rate of the cases described in the *Report* of 1928 was 58 per cent., the mortality in 71 American cases reported by Armstrong (1932) was 37 per cent. Probably an average case mortality rate is about 40 per cent, showing that the disease is a very serious one

PATHOLOGY

The pathology of postvaccinal encephalitis is of great interest, mainly because it shows the disease to be closely related to numerous other conditions characterized by demyelination In Britain the papers attracting main atttention have been those of Turnbull and McIntosh (1926), Perdrau (1928), and Turnbull (1928), and it is on these that the following account is mainly based Numerous other authors have described the pathological features of the condition (Bastiaanse *et al*, 1925, Bouman and Bok, 1927, Lucksch, 1927, Pondman, 1927, McIntosh and Scarff, 1927–8, Bijl, 1928, McIntosh, 1928, [illegible] 1928,
Coyle and Hurst, 1929, Kling, Lon [illegible] erido,
1930, Bastiaanse, 1931, von Herre[illegible] arren,
1931, Globus, 1932, Spielmeyer, 1932, Kokken, 1937, Kaiser and Zappert, 1938, Lamm, 1938, Swan, 1944)

Central Nervous System

Naked-eye, the meninges appear congested Microscopically, there is a mild inflammatory reaction with some hemorrhages, the infiltrating cells are mainly lymphocy[illegible] cells
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5. *Nerve cells* The most characteristic feature is that the cells are usually well preserved, even in areas showing a profuse cellular infiltration

An encephalitozoon-like parasitic cyst was described in the nerve cells of postvaccinal encephalitis cases by Kling, Wassén, and Faahraeus (1932 *a*, *b*), but this observation has not been generally confirmed

6 *Subacute lesions* Subacute cases have been reported where scarring was present, there was also an intense cellular infiltration, and fat-granule cells were present in large numbers in the vessel sheaths (Turnbull and McIntosh, 1926, Perdrau, 1928, Turnbull, 1928).

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EPIDEMIOLOGY

Incidence

One of the most characteristic features of postvaccinal encephalitis is the rarity with which it follows primary vaccination in infancy; the bulk of cases occur in children over 2 years of age. For example, in Holland from 1927 to 1936, Bastiaanse (1937) recorded only 9 cases in children below 2, although there were 116 in those aged 2 to 11. From 1923 to 1927 there was only 1 case of postvaccinal encephalitis in infants under 1 year, although 27,405 were vaccinated. He found that the ratio of cases of postvaccinal encephalitis to vaccinations was 1/2,900 from ages 2 to 11, but only 1/16,000 for those under 2.

In Holland, during the period of high incidence, the commonest age for children to be vaccinated was just before they went to school; accordingly, the majority of cases occurred in those aged 3 to 5. In England, however, if not vaccinated in infancy, the primary vaccination might be carried out at any time later, usually during the school period, the commonest age for postvaccinal encephalitis to develop, therefore, was from 6 to 14. In recent experience this age incidence has been confirmed (Anderson and McKenzie, 1942).

Sex. There seems often to have been a predominance of female cases Thus, in 62 cases cited in the English *Report* (1928) 40 were in females and 22 in males.

Grouping of cases. Certain small groups of cases have, on occasions, been noted e g in London, Bristol, Sheffield, and Wareham in Great Britain, in an American city (Armstrong, 1932); and in Kufstein in the Tyrol (Kaiser and Liedl, 1929) The bulk of cases have occurred sporadically, however, without any apparent connection between each other; familial cases are most unusual.

Geographical Distribution

At first recognized chiefly in Holland, Great Britain, and Germany, it is now known that postvaccinal encephalitis may occur in many other parts of the world. The disease appears to have been very common from 1922 to 1930, and to have become considerably less prevalent of recent years.

The following information on the geographical incidence of postvaccinal encephalitis has been gleaned from numerous original papers (*vide infra*), as well as from special reports (see in particular *League of Nations Report*, 1928; *Report*, 1928, 1929, 1930, *Bull Off int. Hyg publ.*, 1934, 1935, 1936, 1946)

Great Britain Turnbull investigated a case of postvaccinal encephalomyelitis in London in 1912. The results of this investigation were not generally known until, with McIntosh in 1926, he reported 6 further necropsies of similar cases. Three of these cases occurred in 1922, and 3 in 1923, Turnbull recorded a further case in 1928 Numbers of papers and reports have referred to the incidence of postvaccinal encephalitis in Britain (Winnicott and Gibbs, 1926, Martin, 1928, Perdrau, 1928, *Report*, 1928, 1930, 1931, Russell and Oddie, 1928, Taylor, 1929, Roper, 1933).

The official *Report* of 1928 gives the following information regarding the total numbers of cases brought to notice in England and Wales In November and December 1922 there were 11 cases, in the summer and autumn of 1923 there were 50 cases The cases of 1922 occurred in the London area Those in 1923 were widely dispersed over England and Wales, it was possible, however, to define a belt of incidence extending from Exeter and the Bristol Channel, via the Midlands, to Morpeth in Northumberland Cases occurred outside this belt in London and the Home Counties, it was noted that hardly any cases occurred on the coast In 1924 and 1925 there were only a few cases reported From January 1926 to October 1927 the disease became more prevalent, there being 25 cases, of these, 20 occurred in the first three-quarters of 1927, 11 of the 20 cases occurred in 2 groups, namely,

5 in Sheffield, all within 12 days, and 6 in a localized area on the Monmouth–Glamorgan boundary.

The later *Report* (1930) of the Rolleston Committee reported 80 cases occurring between 1 October 1927 and 30 September 1929. Two particular groupings were noted in this set of cases. First, during the last quarter of 1927, 7 cases occurred in Bristol and 2 in the neighboring district of Kingswood, further, 6 of the Bristol cases occurred in an area of 1 square mile. The second remarkable grouping was at Wareham village, where 5 cases of postvaccinal encephalitis occurred. Although there was some other evidence of grouping in various localities, 48 cases were widely scattered over the country.

recorded (*Bull Off. int. Hyg publ*, 1935), while from 1934 (October) to 1935 (October) only 1 case was recorded (*Bull Off. int. Hyg. publ*, 1936). In 1936 there were 5 cases (Morgan, 1938) A later case was that of Sakoschansky and Trenchard (1939).

Holland. Main interest has always centered in the Dutch cases, as the disease is commoner in Holland than elsewhere. Many papers and reports have been published by those who have investigated such cases (Bastiaanse *et al*, 1925; Bastiaanse, Bijl, and Terburgh, 1926, Terburgh, 1927, 1929, 1930, *League of Nations Report*, 1928, *Report*, 1928, Netter, 1929 *c*, Aldershoff, 1930 *a*, Jitta, 1930, 1931; Hekman, 1930, Paschen, 1930, Terburgh, 1930; Bastiaanse, 1931, 1932, 1937).

As regards the number of cases, Jitta (1931) reported that 186 occurred between 1924 and 1931. More recently, Bastiaanse (1937) referred to 125 cases between January 1, 1927 and January 1, 1936. In 1933 there were only 8 cases (*Bull Off. int Hyg. publ*, 1935), and in 1935 only 4 (*Bull. Off. int. Hyg publ*, 1936). It is evident, therefore, that the disease is much less common at the present day than it was from 1925 to 1930 A high incidence of postvaccinal encephalitis in small communities was a characteristic feature of the Dutch cases For example, in 2 large cities the incidence of postvaccinal encephalitis was 1 per 9,000 recently vaccinated persons, but in small towns the rate was 1 case per 63 vaccinations (*Report*, 1928).

The factors causing the high incidence of postvaccinal encephalitis must have been purely local, this was strongly suggested by the fact that a certain preparation of neurovaccine failed to produce any accidents in Spain, but when used for vaccination in Holland a number of cases was recorded (Paschen, 1930).

America. Various reports have referred to the incidence of postvaccinal en- ... Flexner 1929 *a*, *b*, Fulgham 1930, Tuthill, 1930; Wolf ... nd Warren, 1931; Roehm, ... 6, Lancaster, 1938). Bauer was able to find records of 74 cases up to 1935.

Germany. A number of cases has been recorded in Germany, and it would appear that at least 100 cases have now been described (Pette, 1927, Hamel, 1929, 1931, *Report*, 1929, Esser, 1930, Weichsel, 1931, Eckstein, 1929 *a*, *b*, 1932 *a*, *et al*, 1931, *Bull. Off int Hyg publ*, 1934, 1935, 1936 ...

With regard to *Austria*, Redlich (1927), K ... (1929) presented early reports Lately, Kaiser ... a monograph giving a bibliography of Austrian publications, they recorded 240 cases Stiefler (1938) has reviewed the cases occurring from 1930 to 1937.

Norway Holst (1935) refers to some 60 cases of the disease, one-third of which occurred in Oslo, although this town only contains one-tenth of the whole population (see also Holst, 1930, 1939, Ustvedt, 1930, *Bull. Off. int Hyg publ*, 1935). Voss (1938) refers to 80 cases between 1921 and 1937.

Sweden. A number of workers record the occurrence of postvaccinal encephalitis in this country (Kling, Lonberg, and Wassén, 1929; *Report*, 1929, Kling, 1932; Kling *et al.*, 1932 *a*; *Bull. Off. int. Hyg. publ.*, 1934, 1935, 1936, Olin, 1938).

In all the above countries the disease has been recognized quite commonly. In the countries listed below, only comparatively few cases have been recorded.

Australia Lockwood (1931) recorded a case that developed in 1925.

British Guiana. Grace (1929–30) reported a case.

Canada. Gordon (1931) referred to a probable case seen in 1914, and to a later one diagnosed histologically (see also Gordon and Rhea, 1932).

Czechoslovakia Lucksch (1924, 1925, 1926) drew attention to cases occurring in Bohemia from 1923.

Belgium. Probably under 20 cases have been recorded (see van Bogaert and Borremans, 1931; *Bull. Off. int. Hyg. publ*, 1935; Meunier, 1936–7, 1937, Van Den Branden and Nelis, 1936–7; Kokken, 1937).

France. Comby (1907, and see 1926) observed in 1905 and in 1906 what were perhaps the first 2 recognized cases of postvaccinal encephalitis The incidence of the condition has remained very low, comparatively few other papers having appeared (Dévé, 1929, Paisseau and Scherrer, 1929, Pagniez, 1930; Gounelle, 1931, Bertoye and Bouvier, 1932; Imbert, 1935; Giraud *et al.*, 1938, see also *Report*, 1929)

Italy. Taccone (1936) mentioned over 100 cases as having occurred in Italy, more recently Petragnani (1938) recorded 11 cases in 1936 and the first half of 1937.

Mexico Almazan (1936) recorded the occurrence of 8 cases since 1928.

Oceania Clouston and Quin (1937) referred to a case on the island of Nauru (0 15 S, 167 0 E.).

Roumania. Paulian, Arceesco, and Finkelstein (1932) recorded a case.

Russia. Syssine (1930) refers to the occurrence of postvaccinal encephalitis in this country

Switzerland Only a few cases have been reported from this country (see *Report*, 1929, Carrière, 1930, *Bull Off. int. Hyg. publ.*, 1936).

Yugoslavia. The occurrence of cases in this country has been referred to by Yoannovitch (1930)

India Cases have only rarely been recorded, a recent one being that of Nicol (1938) in the Punjab.

Africa. The complication is surprisingly rare in Africa, despite the very large number of vaccinations performed (Davies, 1947)

Relationship to Cases of Smallpox

There does not seem to be any relation between the number of cases of postvaccinal encephalitis and smallpox occurring in a given locality. For example, the *Report* of 1928 states that there were 3 cases of postvaccinal encephalitis in both Gloucestershire and Oxfordshire, yet the former county had 617 cases of smallpox, and the latter none, further, in Worcestershire, with only 1 case of smallpox, there were 7 of postvaccinal encephalitis.

THE RÔLE OF VACCINATION IN THE ETIOLOGY OF POST-VACCINAL ENCEPHALITIS

The Lymph

There has never been reason to blame any particular sample of lymph

It has been suggested that postvaccinal encephalitis only became prevalent after the introduction of vaccine lymph which had at one time in its history been passed through a rabbit (Coplans, King, and Simpson, 1929) However, this does not explain the fact that postvaccinal encephalitis is now comparatively uncommon, although rabbit-passed virus is still in use

Number of Insertions

The later *Report* (1930) of the Rolleston Committee recorded 42 cases primarily vaccinated in 4 insertions, 2 cases in 3 or 4, 13 cases in 3, 7 cases in 2; and only 2 cases that were vaccinated in a single insertion Similar observations were recorded in the first *Report* (1928). It seems evident, therefore, that multiple insertions are to be deprecated, and single to be commended The *Report* of 1928 did actually contain such recommendations.

The Rôle of Primary Vaccination

The great majority of cases occurs after primary vaccination, and not after revaccination For example, in the later *Report* (1930) of the Rolleston Committee, 83 out of 90 cases were in primarily vaccinated persons. In 71 cases in the United States of America only 2 occurred in revaccinated patients (Armstrong, 1932). In Germany Hamel (1929) reported postvaccinal encephalitis to follow 46 primary vaccinations, and only 5 revaccinations In the Edinburgh mass vaccination campaign (1942) 20/23 cases were in persons vaccinated for the first time (Clark, 1944). It has been said that postvaccinal encephalitis following revaccination develops after an incubation period of about 5 days, as compared with the usual 12 days (*Hekman, 1930*).

Effect of Incidence of Vaccination

It is logical to ask whether an increased incidence of vaccination brings in its train an increased number of cases of postvaccinal encephalitis. The figures of the 1928 *Report* gave no direct support for such a relationship Thus, in Gloucestershire 27,800 tubes of lymph were issued to public vaccinators, and 3 cases of postvaccinal encephalitis resulted *In Worcestershire there were 7 cases of* postvaccinal encephalitis (i e., over double the Gloucestershire figures), but only 16,639 tubes of lymph were issued. Further, Oxfordshire had as many cases of postvaccinal encephalitis as Gloucestershire (3), but only 4,100 tubes were issued, as against 27,800 in Gloucestershire.

In apparent contrast to these English figures, Terburgh (1929) in Holland cites the following evidence that there is a relationship between the number of cases of postvaccinal encephalitis and the incidence of vaccination 188,000 vaccinations in March yielded 46 cases of postvaccinal encephalitis, 99,000 vaccinations in April yielded 14 cases of postvaccinal encephalitis, 46,000 vaccinations in May yielded 2 cases.

The question of the connection between the incidence of vaccination and the occurrence of postvaccinal encephalitis must, therefore, remain unsettled. There is no doubt, however, that groups of cases are now seldom seen except associated with a mass vaccination campaign

Ratio of Cases of Postvaccinal Encephalitis to Vaccination

In England and Wales the ratio of cases of postvaccinal encephalitis to cases of vaccination was 1/49,000, whereas in Holland it was 1/4,000 (*Report*, 1928), this suggested the presence in Holland of some local factor very potent in the causation of postvaccinal encephalitis In recent experience the incidence in Edinburgh in 1942, for instance, was 1 20,000 (Clark, 1944), in Glasgow 1/70,000 (Anderson and McKenzie, 1942), and in Fife 1 7,000 (Fyfe and Fleming, 1943).

PREVENTION OF POSTVACCINAL ENCEPHALITIS

The Rolleston Committee (*Report*, 1928) recommended that a trial be made of vaccination in one insertion, with a minimum of injury, and without crosshatching. They recommended that persons should be vaccinated *in infancy, between 2 and* 6 months, revaccinated at 5 to 7 years of age, and again at 14 to 16 years The

great point is to avoid primary vaccination of children over the age of infancy; for it will be recalled that the disease is very rare in infants, occurring mainly in those of school age. They also recommended that public vaccinators should inspect their patients 7 to 10 days later, and also after 14 to 17 days

SPECIFIC THERAPY

Certain authors have reported beneficial results to follow the administration of serum of persons recently vaccinated (Horder, 1929, Hekman, 1929, 1930, Roper, 1933, see also Paschen, 1930; Netter, 1931; Davidson and Thomas, 1942; Dunn, 1943; Fyfe and Fleming, 1943; see also Loutit and McClean, 1945). Horder and Roper administered the serum intrathecally, and Hekman intravenously. Béclère (1931) recommended serum of a vaccinated calf.

ATTEMPTED ISOLATION OF THE ETIOLOGICAL AGENT OF POSTVACCINAL ENCEPHALITIS

Writing in the *Report* of 1928, McIntosh and Blaxall recorded the presence of vaccine virus in the brains of 3 cases. At first this virus was maintained by rabbit passage, but later it could be passed through calves (see also Turnbull and McIntosh, 1926). Aldershoff (1930 *b*) was also able to isolate vaccinia virus from the central nervous system of fatal cases.

Certain authors have recorded the presence of vaccinia virus in the cerebrospinal fluid of up to a third of cases of postvaccinal encephalitis, although absent from that of normal vaccinated persons (Gildemeister, 1929, Eckstein, Herzberg-Kremmer, and Herzberg, 1930 *a*; Herzberg-Kremmer and Herzberg, 1930, 1932).

Despite these positive results, Gordon (*Report*, 1928) was unable to find the virus in the central nervous system of 5 cases of postvaccinal encephalitis, nor did suspensions of such tissues immunize rabbits against vaccinia Others have also failed to isolate virus (Bastiaanse *et al.*, 1925; Bijl, 1926, 1927; Kraus, 1927, Esser, 1930) This failure has been attributed to a virucidal substance in the brain (Bijl and Frenkel, 1929). Another possible explanation is that the condition of "autosterilizable neuroinfections" of French authors occurs; that is to say, that the host reacts to the presence of vaccinia so efficiently that the virus is all destroyed, and none can be isolated from the brain.

In conclusion, vaccine virus is certainly the only one which has been isolated from cases of postvaccinal encephalitis, but this has not been accomplished with sufficient regularity to bear much weight Further, it is probable that vaccine virus normally enjoys a wide distribution throughout the tissues of the vaccinated person, so that the finding of vaccine virus in the brain or cerebrospinal fluid of cases of postvaccinal encephalitis loses most of its apparent significance.

THEORIES OF THE ETIOLOGY OF POSTVACCINAL ENCEPHALITIS

A large number of theories has been put forward to explain the etiology of postvaccinal encephalitis, certain established facts should be borne in mind when considering these. First, postvaccinal encephalitis follows vaccination at an interval of about 12 days Second, the virus of vaccinia has been isolated from the brains of cases on a few occasions, this loses much of its importance, however, in view of the fact that the virus is known to disseminate in the body of vaccinated persons who do not develop postvaccinal encephalitis Third, a disease, similar histologically, follows measles, antirabies treatment, and perhaps other conditions Fourth, the histological picture of certain other demyelinating diseases is very similar to that of postvaccinal encephalitis

The various theories that have been advanced will now be discussed *seriatim* Information on the etiology of other demyelinating processes should also be read, e g., neuroparalytic accidents after rabies (see Ch LXXIX), and measles encephalitis (see Ch. XX).

1 *Cerebral vaccinia* It is most natural to suppose that postvaccinal encephalitis may be due to an invasion of the cerebral tissue by the vaccine virus itself, the strain being endowed perhaps with special neurotropic tendencies In favor of this theory, the incubation period is constant at about 12 days—a period similar to that of smallpox. More important, however, is the fact that vaccinia is the only virus ever isolated from the central nervous system in postvaccinal encephalitis Further, it should be noted that similar cases may occur in the course of smallpox (see p 290). Another point in favor is the fact that benefit has been produced by the serum of recently vaccinated persons On the experimental side McIntosh supported the theory because of similarities which he detected between the lesions of postvaccinal encephalitis and those produced, experimentally, by injection of rabbits and monkeys with vaccinia Eckstein and also Clearkin obtained results which support this theory, for they found changes reminiscent of postvaccinal encephalitis in monkeys inoculated by various routes (see p 325). McIntosh appended a minority report to that of the Andrewes Committee (*Report*, 1928), in which he supported this theory (*see also 1930, McIntosh and Scarff, 1927–8*). The theory of cerebral vaccinia has been upheld by others (Netter, 1929 *b*, 1931; Eckstein, Herzberg-Kremmer, and Herzberg, 1930 *a*, *b*).

There are, however, certain objections to this theory.

(*a*) Vaccination has been carried out for generations, yet apparently this complication has only recently been recognized

(*b*) The presence of virus in the brain does not necessarily indicate that it is playing any pathogenic rôle, as it is known to disseminate widely in the bodies of normal vaccinated experimental animals and human beings

(*c*) Even so, many authors have quite failed to isolate vaccinia virus from the brain or cerebrospinal fluid of cases

(*d*) The histological picture of postvaccinal encephalitis is generally held to be quite unlike that produced by intracerebral injection of vaccinia in animals

(*e*) There has never been evidence adduced to incriminate any particular batch of vaccine lymph No special tendency to neurotropism has been found in the virus in specimens of lymph giving rise to postvaccinal encephalitis On one occasion neurovaccine was used for vaccination in Spain, without production of postvaccinal encephalitis, the same vaccine was brought to Holland, where the usual high incidence of postvaccinal encephalitis occurred (Paschen, 1930).

(*f*) Some authors have quite failed to produce encephalitis in experimental animals after inoculation of the skin, despite maneuvres designed to cause the virus to localize in the nervous tissue (see p 318).

(*g*) Conditions similar to postvaccinal encephalitis follow infection by other agents, e g., measles virus, and during antirabies treatment It is difficult, then, to claim any specificity for vaccinia virus.

2 *Activation theory* A theory which has been widely held is that vaccination serves to activate some other virus which is lying latent in the body. Two viruses, in particular, have been investigated with regard to the possibility of their being stimulated by vaccination. For example, Gordon, as well as Perdrau, has shown experimentally that there is no evidence that herpes virus can be stimulated by vaccinia to produce a condition resembling postvaccinal encephalitis. Hurst and Fairbrother (1931) found no evidence to suggest that poliomyelitis virus could be activated by vaccinia

As it has not proved possible to incriminate any known virus, recourse has been

1933–4, 1935). We do not, however, feel able to support this theory, as there is virtually no positive evidence in its favor.

3. *Theory of fortuity.* It has been suggested that postvaccinal encephalitis is none other than encephalitis lethargica or polioencephalitis. For example, Levaditi, Nicolau, and Sanchis-Bayarri (1927) suggested that postvaccinal encephalitis is caused by vaccinia "lighting-up" the causal agent of encephalitis lethargica. They considered that, clinically, certain cases of the 2 diseases are alike, histologically also there are similarities; and there is difficulty in isolating a causal agent in both conditions. There are certain serious objections to this theory:

(*a*) The constancy of the incubation period argues against the cases being fortuitous examples of another known nervous disease.

(*b*) [illegible] usually distinguishable,

(*c*) [illegible] itis, one would expect that the characteristic virus would be isolated. The histological examination can also distinguish between the 2.

(*d*) With regard to both, there has seldom been any definite coexistence of postvaccinal encephalitis and typical cases of either polioencephalitis or encephalitis lethargica. Studies of the relative incidences and mortalities of the 3 conditions have not disclosed any relationship that would suggest that postvaccinal encephalitis is caused by polioencephalitis or encephalitis lethargica.

4. *Other infective agents.* (*a*) It has been suggested that postvaccinal encephalitis is caused by a virus which finds its way into the site of vaccination at the time of operation, or later (see, e.g., Aldershoff, 1935). The virus must presumably, therefore, either be present on the patient's skin, or else in the lymph used as inoculum. There is absolutely no evidence that such a virus exists, and the theory is a most unlikely one. It should be noted that although lymph has been found contaminated with foot-and-mouth virus (Van Heelsbergen, 1931) there is not the slightest evidence that such an occurrence is anything but the greatest rarity. At any rate, it is unlikely that the virus of foot-and-mouth disease could cause such a condition as postvaccinal encephalitis.

(*b*) It has been suggested that the cause of postvaccinal encephalitis is a *Pasteurella* organism showing bipolar staining (Aldershoff and Pondman, 1928). They claim to have isolated this organism from the vaccine lymph used, and the nasal discharge, in 1 case.

In rabbits they claimed that the intracerebral inoculation of vaccinia activated a bipolar organism already present (probably in the nasal passages), so that it could be isolated from the brain. Pette (1928) showed experimentally that rabbits inoculated with vaccinia virus might succumb to a *Pasteurella* infection. The vaccination activated these organisms lying in the nasal passages.

(*c*) Aldershoff (1930 *a*) does not believe that postvaccinal encephalitis is caused by vaccinia, and has blamed a *Monilia* organism.

(*d*) Heerup (1936) postulated that a primary focus of infection in the tonsils gave rise to a state of toxemia, and that this was the essential factor in the etiology of postvaccinal encephalitis.

(*e*) In one case it appeared as if hereditary syphilis acted as the predisposing cause (Murano, 1937).

(*f*) Caminopetros *et al.* (1938) claim to have isolated a hitherto undescribed virus, by animal inoculation, from the cerebrospinal fluid of one case of postvaccinal encephalitis.

5. *Theory of allergy.* Glanzmann (1927) suggested that postvaccinal encephalitis may be due to a local anaphylactic reaction in the central nervous system, and this theory has been supported by others (Mikulowski, 1927, Euzière and Pagès, 1928, van Bogaert, 1932). Evidence of an allergic state was obtained in one case by

McClure (1930), whose patient developed urticaria shortly after vaccination, postvaccinal encephalitis following in 10 days' time.

Finley (1938) has recently strongly supported the allergic theory He quotes von Pirquet's (1907) description of the primary lesion of vaccination as follows The lesion is composed of 2 parts (*a*) the papule—regarded as nonspecific, and (*b*) the areola—a specific virus response appearing about the 4th day, this areola rapidly increases in size from the 8th day, and is maximal on the 11th to 12th von Pirquet held that the areola was the result of the interaction of antibodies and virus in the neighborhood of the papule Finley's main argument is based on the presumption that antigen-antibody interaction occurs in other parts of the body, as well as the skin lesion, at this time. In evidence he states that the temperature rises, further, the local and general vaccinial eruptions that may be noted occur at the acme of the primary lesion, he emphasizes the constancy of the incubation period of postvaccinal encephalitis, which is slightly shorter after revaccination. Finally, he recalls that the brain of experimental animals acquires a local immunity after vaccination, and that the brain of a case of postvaccinal encephalitis neutralized vaccinia virus (Bijl and Frenkel, 1929). All these facts, he suggests, indicate that an antigen-antibody reaction may occur elsewhere in the body, including the brain. Granted that such a reaction does occur in the brain, it would probably take the form of a vascular thrombosis, and this we know produces demyelination Here we may quote 2 reports of experimental work

First, Alexander and Campbell (1937) sensitized guinea-pigs by a single injection of horse serum intraperitoneally, later, the "shock-provoking" dose was given directly into the brain Anaphylactic lesions were produced in the brain, having the following characteristics there were vascular thromboses and hemorrhages, necroses, demineralization, infiltration with leukocytes, microglia, oligodendroglia and astrocytes, the areas immediately peripheral to these lesions were anemic. They found similar, but very much smaller, lesions in unsensitized animals given a single intracerebral dose

Secondly, Abell and Schenck (1938) have employed the transparent chamber technique, whereby the living vessels in the rabbit's ear may be examined They first sensitized rabbits to horse serum, and later injected antigen intravenously, or into the transparent chamber. The second injection might be followed by a contraction of the arterioles, obliteration of the lumina, and cessation of blood flow. Microscopically, there was adherence of leukocytes to the vessel walls, and emigration through the walls, leukocytes were seen to adhere in the form of clumps. They reported that a similar reaction might follow the first injection These intravascular lesions may be considered as the preliminary stages of a thrombosis

Conclusions

It must be realized that postvaccinal encephalitis is only one of a number of diseases which all present a comparable histological appearance, demyelination being the main feature For example, similar conditions may complicate measles, smallpox, influenza, antirabies treatment, and perhaps varicella, these have all been referred to elsewhere Perivascular demyelination can also be produced experimentally by injection of coagulants and oils, which cause intravascular thrombosis (Putnam, McKenna, and Morrison, 1931, Putnam, 1935; Hoefer, Putnam, and Gray, 1938) In addition, we should mention that certain "spontaneous" conditions also resemble postvaccinal encephalitis, e g, Devic's disease, Schilder's disease, acute disseminated encephalomyelitis, and disseminated sclerosis Of recent years, much active work has been carried out on the etiology of demyelinating diseases Personally we feel that such work may eventually furnish the solution to the etiology, not only of disseminated sclerosis itself, but also of postvaccinal and the other infective forms of encephalomyelitis It is outside the scope of this book to describe this work in detail (see Putnam, 1935–6, 1937). We may, however, mention that

Hurst (1942) has suggested that the demyelination induced by chemicals is produced by repeated periods of anoxia. He has produced a continuous range of lesions ranging from necrosis to demyelination merely by varying the inoculum. It is probable that a number of agents, some viruses, some chemical substances, some bacteria, and some as yet unidentified, have the property of causing demyelination. Some of these agents may cause demyelination by a direct action on the myelinated tissue, and it is possible that the viruses act by causing the blood to clot more readily, or by inducing anoxia. The resultant vascular thromboses produce the characteristic histological picture of demyelination. It is possible that the primary factor in causing the thrombosis may be of an allergic character.

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SECTION 4. ANTHROPOD-SPREAD AND OTHER TROPICAL INFECTIONS

CHAPTER XXXVI

RIFT VALLEY FEVER

Rift Valley fever or enzootic hepatitis was first described by Daubney, Hudson, and Garnham in 1931. These workers found the disease in Kenya Colony among sheep, and at the same time recorded its experimental transmission to man. In all probability the condition was noticed earlier by Montgomery (1913), who recorded an outbreak of a similar disease causing a 90 per cent mortality among lambs at a government farm at Naivasha in Kenya. The 1930 outbreak, which was described by Daubney and his coworkers, occurred on a farm situated at an altitude of 5,500 to 6,000 feet extending northwards from the shores of Lake Naivasha in the Rift Valley. About 3,500 lambs and 1,200 ewes died from the disease, and the mortality attained a figure of 95 per cent. among the 3 to 7 days' old lambs (see also Findlay and Daubney, 1931).

Rift Valley fever has been noticed in Kenya, Uganda, Anglo-Egyptian Sudan, French Sudan, and French Equatorial Africa (Findlay, Stefanopoulo, and MacCallum, 1936).

THE DISEASE IN MAN

Daubney, Hudson, and Garnham (1931) demonstrated the infectivity of Rift Valley fever virus for man by inoculating a human subject with a filtrate derived from infected sheep tissue. Later, Findlay (1932 *a*) recorded that many of the natives employed in herding sheep during the period of the enzootic also suffered from a short febrile condition resembling the experimental disease in man. Then, Schwentker and Rivers (1934) recorded a fatal case in a laboratory worker who contracted the disease and died following complication with thrombophlebitis. Kitchen (1934) described another laboratory infection, and subsequently Francis and Magill (1935) and Findlay (1932 *a*) drew attention to 6 other cases that were acquired in a similar fashion. In all, nearly 20 cases of laboratory infection have been recorded, even from virus passed hundreds of times in mice (Sabin and Blumberg, 1947). Curasson (1934) has suggested the possible occurrence of the disease in the French Sudan. The general course of the malady is that of a mild febrile illness and, according to Daubney, Hudson, and Garnham, at least 200 human cases have occurred in British East Africa, without complications or death.

Clinical Features

After an incubation period of 5 to 6 days, the symptoms of the disease develop suddenly. A wide variety of clinical manifestations is liable to be present, such as general malaise, headache, nausea, vomiting, shivering, rigors, shooting pains in the muscles and joints, particularly affecting the shoulder region and back, a sense of fullness over the liver, tenderness of the eyeballs, photophobia, mental confusion, vertigo, and epistaxis. The pyrexia varies from about 101 to 103° F., is of the so-called "saddle-back" type resembling that recorded in dengue, and may occasionally reach 105–6° F., the pulse rate being also increased. Usually, the disease only lasts for a few days and the convalescent period is short. Relapses have been reported (see Francis and Magill, 1935).

Clinical Pathology

The most characteristic feature is the presence of a leukopenia. A figure of 3,720 w.b.c. per *cmm* has been recorded by Francis and Magill, and the decline principally affects the polymorphonuclear leukocytes. Findlay (1932 *a*) has recorded the presence of occasional myelocytes and polymorphonuclear leukocytes possessing vacuolated nuclei. He also states that the leukopenia may persist into convalescence. Throughout the disease the patient's urine may appear to be normal in its color and constituents, containing neither blood, bile, nor casts. Findlay (1932 *a*), however, has mentioned a case in which the urine was of a deep yellow color but was not pathogenic for mice. Blood, or throat washings derived from patients during the height of the illness are infective for mice.

Pathology

The only fatal case on record is that reported in a laboratory worker who died from venous thrombosis, 45 days after the onset of infection. The acute stage of his illness only occupied the first week. At autopsy, clots were present principally in the inferior vena cava, and to a lesser extent in the saphenous and femoral veins. Chronic pleuritis together with old pulmonary infarcts and emboli were present in both lungs. The liver showed no abnormalities, and the virus could not be recovered from postmortem tissue. This was doubtless due to the active stage of the disease having subsided some considerable time prior to death (Schwentker and Rivers, 1934).

THE NATURAL DISEASE IN SHEEP

This is of a variable character, and the following signs have been recorded: vomiting, diarrhea, melena, mucopurulent nasal discharge, loss of appetite, abortion in pregnant animals, unsteady gait, and death usually within 24 hours of the onset of symptoms. The temperature is often normal.

The liver. Focal necrosis of this organ is the most striking feature of the disease. Small white foci of necrosis about 1 *mm* in diameter appear beneath the capsule of the liver, frequently surrounded by pin-point hemorrhages. Necrotic foci are evenly distributed throughout the organ, and when numerous they tend to coalesce, forming a diffuse necrotic lesion. There is, however, no sign of demarcation of necrotic areas from surrounding liver tissue. The liver is usually not increased in size but is occasionally engorged with blood.

The spleen is not enlarged, but shows numerous subcapsular pin-head hemorrhages, especially at the free border of the organ.

The kidneys. Hemorrhages and capillary arborescence have sometimes been recorded.

The lungs. Small, slightly raised areas which are oval in shape and an inch or more in diameter occur on the surface of the organ.

Circulatory system. Subpericardial ecchymoses are common in the region of the coronary grooves or the ventricles of the heart. The mesenteric vessels are always strongly injected and engorged with blood.

Lymphatic glands in the mesentery may be enlarged and show cortical necrosis.

Alimentary tract. The lesions here are of variable character, the principal changes affecting the lower intestine being hyperemia, congestion, and in severer cases, hemorrhagic enteritis.

CHARACTERISTICS OF THE VIRUS

Pathogenicity to Animals

Other than its infectivity for the lamb, sheep, and the human subject, the virus has been shown to be pathogenic to a number of other species of animals (see

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brane, and the appearance of acidophilic material within the nucleoplasm In fresh tissue the latter may be stained by vital dyes such as dilute water-soluble eosin, methylene blue, neutral red, or phloxine red Janus green B and osmic acid exert no effect upon them Findlay states that the intranuclear inclusions of Rift Valley fever are dissolved by 0 1 per cent. acetic acid or 1 per cent ammonia; moreover, he adds that their existence in fresh tissue militates against their being artefacts in origin. Chemical tests have failed to disclose the nature of their composition, as they are neither blackened by osmic acid, nor can they be stained with sudan III, Scharlach R, or Nile blue sulfate in frozen sections They give a negative reaction for chromatin by the Feulgen technique, and also a negative Bensley-MacCallum for masked iron. The following is a list of stains used by Findlay for demonstrating intranuclear inclusions of Rift Valley fever iron hematoxylin and eosin, Giemsa, eosin and methylene blue, phloxine and methylene blue, resorcin fuchsin, phosphotungstic acid hemotoxylin, Mallory's stain, Castañeda's stain, neutral red and neutral gentian violet.

For work bearing on micro-incineration studies carried out in Rift Valley fever the reader is referred to the studies of Horning (1934).

Neurotropic Strain of Rift Valley Fever Virus

Mackenzie and Findlay (1936) passaged the virus 30 times intracerebrally through mice. By this method they succeeded in producing a neurotropic strain of virus which possessed a fixed affinity for nervous tissue, and such a strain, when injected intracerebrally in mice, produced encephalomyelitis without liver necrosis. Rhesus monkeys were also infected with this strain by intracerebral or intranasal inoculation. Rabbits and guinea-pigs were apparently insusceptible (Mackenzie, Findlay, and Stern, 1936)

Morphology, Size, and Filtrability of the Virus

This is one of the smallest viruses known, and some of the earliest experiments indicated this fact Thus Daubney and his colleagues found that it passed through the Pasteur-Chamberland L_{11} bougie, and occasionally even the L_{13} size. It also passed through the Berkfeld V, N, and W grades of candle at a pressure of 630 mm Hg (Findlay, 1932 a) Subsequently its size was more accurately measured by Broom and Findlay (1933), who filtered it through Elford's gradocol membranes and estimated its dimensions to lie between 23 and 35 mμ According to these figures, therefore, the virus is well beyond the limits of visibility of the ordinary microscope, and is even too small to be photographed by the ultraviolet light instrument (Findlay, 1933) Likewise all attempts to demonstrate the virus by staining have been without success (Mackenzie, 1933).

Cultivation

Cultivation was first performed by Mackenzie (1933), who used the method of Li and Rivers (1930), and employed a culture medium consisting of minced chicken embryo fragments suspended in Tyrode's solution (see Ch. XII) Cultures were put up in 5 c c Erlenmeyer flasks containing 5 c c of extract, incubated at 37° C for 3 days, and subcultivated by transferring 0 2 c c of the growth to a fresh medium In this way Mackenzie cultivated the virus through 13 passages, without either diminishing the infectivity of the material or altering its characteristics Saddington (1934) has also succeeded in cultivating the virus in tissue cultures

Preservation of Virus

Kitchen (1934) has preserved it for at least 8 months by mixing blood from ill mice with normal serum diluted 1/10, and by then desiccating the mixture *in vacuo*. During the course of our own studies on this virus we have preserved it by desic-

Findlay, 1932 *a*). In rhesus monkeys it produces a nonfatal illness, followed by recovery and protection from further attacks for at least 6 months afterwards.

The African monkeys, *Cercopithecus callitrichus*, *Erythrocebus patas*, and *Cercocebus fuliginosus*, fail to exhibit a febrile reaction when inoculated with Rift Valley virus, although it may persist for a few days in their blood Findlay (1932 *b*) pointed out that the relative insusceptibility of these species to infection was not due to the presence of antibodies in their blood, since antibodies only developed after inoculation South American monkeys, such as *Cebus fatuellus*, *C. chrysopus*, *Hapale jacchus*, and *H. penicillata*, were more susceptible and showed a febrile reaction following injection The virus can also be recovered from the blood of these species during a pyrexial period, and immune bodies are demonstrable in the blood of capuchin monkeys 14 days after injection. Cats exhibit a transient febrile illness. The virus is highly pathogenic and proves invariably lethal to mice, field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), dormice (*Muscardinus avellanarius*), and golden hamsters (*Cricetus [mesocricetus] auratus*). In rats the virus causes a 50 per cent. mortality, and is of about the same degree of pathogenicity to the ferret (see Francis and Magill, 1935). The cow, goat, and gray squirrel suffer from a severe but nonfatal malady. Rabbits appear to be resistant, but may harbor the virus for a short time in their blood after infection. The guinea-pig, mongoose, hedgehog, tortoise, frog, hen, pigeon, and parakeet are resistant. Susceptible animals can be infected by any route of inoculation, and in pregnant animals the virus has been shown to be capable of passing through the placenta into the fetal circulation.

Morbid Histology of the Infected Liver

Lesions present in the sheep, goat, rat, mouse, monkey, squirrel, hamster, vole, and wood mouse have been carefully studied by Findlay (1933). The changes are either those affecting the cytoplasm or the nuclei of liver cells, and are as follows

Changes in the cytoplasm.

The initial lesion is focal necrosis of the liver, which may affect any area, but in the rhesus monkey it tends to be confined to the mid-zonal area of the lobule *In lambs, mice, rats, and other small rodents no such distinction* is possible, owing to the rapidity with which the infection spreads through the whole organ. For example, in [illegible] liver tissue left. [illegible] necrosis oc[illegible] bodies within the cytoplasm of individual liver cells. These structures are often surrounded by a clear space and may even be extruded from the liver cell, they tend to be best developed in the most resistant animals such as the adult sheep and goat, whereas they are less well marked in the susceptible mouse. Fatty degeneration and pigmentation are only occasionally seen in the rhesus monkey and are absent in other species *In the case of mice, 48 hours after infection the whole structure* of the liver lobule is destroyed, nuclei show karyorrhexis, the cytoplasm becomes vacuolated and contains a few remnants of mitochondria and nuclear chromatin. [illegible] apparatus were also investigated by Findlay, [illegible] the purpose, and found that all traces [illegible] liver cells at the end of 48 hours in infected mice.

Nuclear changes.

These changes in liver cells are a characteristic feature of the disease. They were originally observed *by Daubney and his coworkers, and later extensively* reinvestigated by Findlay (1932 *a*) The essential changes are margination of the chromatin which becomes distributed inside the periphery of the nuclear mem-

moreover, the characteristic liver necrosis evident in Rift Valley infection of laboratory animals bears a striking resemblance to human liver necrosis caused by yellow fever virus There is, however, no reason to believe that Rift Valley fever produces extensive liver lesions in man, at least, in those cases which have been quoted in the literature, it has not caused sufficient damage to be recognizable *clinically*.

Dengue Fever

Findlay (1932 c) investigated this problem and found that these conditions were entirely distinct and separate pathological entities. Thus he successfully demonstrated, by means of cross neutralization tests, that the monkey *Silenus rhesus* when immunized against dengue was not immune to Rift Valley virus In the same way, monkeys immunized against Rift Valley fever could be infected with dengue virus Also, mice injected with dengue virus were not immune to Rift Valley fever virus

Yellow Fever

The points of similarity and dissimilarity existing between this and Rift Valley fever virus are summarized in an excellent account of the subject in the work of Findlay and MacCallum (1937) The features of differentiation may be summarized thus Both viruses are exceedingly small in size, but yellow fever virus, which measures 18 to 27 mμ (see Broom and Findlay, 1933, and Bauer and Hughes, 1935), is the smaller of the two, however, Findlay and MacCallum (1937) have accepted these figures with reserve, in view of the margin of error involved when estimating the size of the smaller viruses by ultrafiltration Rift Valley fever virus is highly infective to sheep, goats, and small rodents, whereas yellow fever virus does not attack these species *In vitro* virus neutralization tests performed with immune sera derived from either condition have failed to reveal the existence of cross reaction between these agents *In vivo* tests have shown that monkeys recovering from Rift Valley fever are susceptible to yellow fever virus and vice versa, moreover a laboratory worker who contracted an attack of yellow fever, 3 years later developed Rift Valley fever

These viruses have many features in common, however, and clinically Rift Valley fever resembles mild yellow fever

Certain species of monkey appear to exhibit the same degree of susceptibility to both infections, according to Findlay (1932 a, 1933) In liver tissue, acidophilic intranuclear inclusions as well as Councilman lesions appear in both diseases Likewise, virus possessing neurotropic properties is obtainable from either agent by repeated intracerebral passage (see MacKenzie, Findlay, and Stern, 1936)

Both yellow fever and Rift Valley fever confer lasting immunity to man following one attack Like yellow fever, Rift Valley fever appears to be transmitted by mosquitoes of the aedes genus (Daubney and Hudson, 1933), and Daubney and Hudson (1932) also showed that the virus was able to survive for 7 days in the engorged nymph of the species *Rhipicephalus appendiculatus* In view of these findings, Findlay and MacCallum (1937) have ventured to suggest that both viruses may have originated from some common ancestral form, although geographically their distributions vary The classification of these two diseases has also been discussed by Broquet (1932)

Influenza

The difficulty of differentiating Rift Valley fever from acute influenza on purely clinical grounds has been emphasized in a report by Francis and Magill (1935) Both diseases exhibit many features in common, for example, the abrupt onset, short incubation period, aches and muscular pains, together with a marked leukopenia

cating mouse liver tissue *in vacuo* over calcium chloride, according to Craigie's method (1931). Findlay (1932 *a*) showed that lamb's blood, to which 0.5 per cent. carbolic acid was added, stored at 4° C, retained its infectivity for mice for 6 months. After 8 months' in oxalate-carbolic-glycerol there was some loss of virulence.

Resistance to Heat

Virus in blood is killed in 40 minutes at 56° C. (Findlay, 1932 *a*).

IMMUNITY REACTIONS IN MAN AND ANIMALS

Complement fixing and virus neutralizing antibodies develop in the first week of illness, and increase markedly in the second week (Broom and Findlay, 1932; Schwentker and Rivers, 1934; Findlay, 1936). Virus neutralizing antibodies have been described 12 years after recovery from a laboratory infection (Sabin and Blumberg, 1947).

Apparently neutral mixtures can be reactivated by dilution, or inoculation nasally instead of peritoneally (Findlay, 1936). The immunization of mice against Rift Valley fever virus has been described by Mackenzie (1935).

An Interference Phenomenon

Findlay and MacCallum (1937) have described the existence of a most interesting phenomenon relating to the apparently antagonistic action of Rift Valley fever and yellow fever viruses when introduced simultaneously into the same animal They have shown that the inoculation of a mixture of both viruses into rhesus monkeys results in protection of 7 out of 10 animals Likewise, a single inoculation of mice with neurotropic yellow fever virus and pantropic Rift Valley fever virus delays their death and even saves a few from it. The protective action is absent, however, if Rift Valley fever virus is injected 24 hours previously. The effect of substituting fowl-pest virus (which is nonpathogenic to mice) was also tried in place of yellow fever virus, but this failed to afford the same degree of protection against Rift Valley fever infection.

In explanation of these findings, Findlay and MacCallum have suggested that, when certain cells are occupied by an actively multiplying virus, they cannot be invaded by other virus particles. They also draw an analogy between this phenomenon and the occurrence of a somewhat similar phenomenon in respect of plant viruses. In the case of plants, it has been shown that a feebly pathogenic agent will afford protection to a plant when infected with a more virulent one, provided that the two viruses are generically related, see Thung (1931), Salaman (1933), Kunkel (1934), Holmes (1934), Ainsworth (1934), Caldwell (1935), McKinney (1935), and Price (1935) The interference phenomenon occurs also in poliomyelitis and influenza (Chs. LXXXI and LIX)

LABORATORY DIAGNOSIS OF RIFT VALLEY FEVER

During a febrile attack the patient's blood should be withdrawn and inoculated subcutaneously or intraperitoneally in a mouse If the animal dies, its liver and blood should first be examined to exclude the presence of bacteria, by aerobic and anaerobic methods of cultivation If the result is negative, then histological sections of liver should be stained and searched for presence of intranuclear inclusion bodies and other changes characteristic of Rift Valley fever. Serum may be injected cerebrally in mice Acute and convalescent phase serum should be tested for the existence of virus neutralizing antibodies

RELATIONSHIP TO OTHER VIRUS DISEASES

Rift Valley fever in man is of interest on account of its clinical similarities to dengue fever on the one hand and epidemic influenza on the other. Pathologically,

moreover, the characteristic liver necrosis evident in Rift Valley infection of laboratory animals bears a striking resemblance to human liver necrosis caused by yellow fever virus There is, however, no reason to believe that Rift Valley fever produces extensive liver lesions in man, at least, in those cases which have been quoted in the literature, it has not caused sufficient damage to be recognizable clinically

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These viruses have many features in common, however, and clinically Rift Valley fever resembles mild yellow fever.

Certain species of monkey appear to exhibit the same degree of susceptibility to both infections, according to Findlay (1932 *a*, 1933) In liver tissue, acidophilic intranuclear inclusions as well as Councilman lesions appear in both diseases Likewise, virus possessing neurotropic properties is obtainable from either agent by repeated intracerebral passage (see Mackenzie, Findlay, and Stern, 1936).

Both yellow fever and Rift Valley fever confer lasting immunity to man following one attack Like yellow fever, Rift Valley fever appears to be transmitted by mosquitoes of the aedes genus (Daubney and Hudson, 1933), and Daubney and Hudson (1932) also showed that the virus was able to survive for 7 days in the engorged nymph of the species *Rhipicephalus appendiculatus*. In view of these findings, Findlay and MacCallum (1937) have ventured to suggest that both viruses may have originated from some common ancestral form, although geographically their distributions vary. The classification of these two diseases has also been discussed by Broquet (1932)

Influenza

The difficulty of differentiating Rift Valley fever from acute influenza on purely clinical grounds has been emphasized in a report by Francis and Magill (1935). Both diseases exhibit many features in common, for example, the abrupt onset, short incubation period, aches and muscular pains, together with a marked leukopenia.

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CHAPTER XXXVII

DENGUE FEVER

Historical

According to Hirsch (1883) this disease has received many different names Thus the Spaniards called it *dengue*, corresponding in meaning to the French word *minauderie* and the English word *dandy* The word *colorado* was also applied by the Spaniards as a descriptive term The English and Americans have called it *break-bone* and *broken-wing*, the French *giraffe* and *bouquet* (whence the English corrupt *bucket*), and the Brazilians have named it polka fever.

Hirsch states that the earliest accounts of dengue fever were those given by Galberti of Egypt in 1779, by Bylon of Batavia in 1779, and by Rush of Philadelphia in 1780. Later, the disease appeared in the West Indies and shores of the Gulf of Mexico, where it was called dengue fever by the local physicians It was not, however, until dengue broke out in the Eastern Hemisphere in 1871–3 that the disease received universal recognition as a distinct clinical entity.

It should be mentioned, however, that the accurate clinical diagnosis of dengue fever is by no means always an easy matter. For example, sporadic atypical cases of dengue may sometimes present clinical signs and symptoms simulating a variety of other febrile illnesses, e g , pappataci fever, malaria, mild yellow fever, and Rift

golaise are no longer regarded as separate entities but accepted as atypical cases of dengue fever

years a number

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end of this chapter.

Dengue is now known to be transmitted by *Aedes aegypti* (*Stegomyia fasciata*), the mosquito that is also responsible for the spread of yellow fever. Since both diseases are disseminated by a common insect vector, the precise geographical distribution of each malady, and likewise of *A. aegypti*, becomes a matter of considerable importance, especially with regard to modern air travel.

Lumley (1943) has published a useful review of the disease

EPIDEMIOLOGY

The rapidity with which dengue fever spreads is popularly regarded as being second only to that of epidemic influenza. For example, Armstrong (1923) states that at Austin, Texas, in 1885, out of 22,000 persons, 16,000 were attacked At Cairo in 1880 four-fifths of the inhabitants were affected, and during the 1818 epidemic at Lima in Peru only a few of the 70,000 inhabitants escaped (Hirsch, 1883). Armstrong (1923) records an outbreak of dengue at Monroe, La , in 1922, among the poorer members of the Negro community, during which 1,000 inhabitants were attacked. Millous (1929) reported an outbreak of 1,000 cases in Cochin-China in 1927, and Hargrave (1931) a similar epidemic in American Samoa, during which 2,842 cases were observed in 2 months

The importance of dengue from the standpoint of military hygiene can be appreciated, since large numbers of soldiers or sailors are liable to be affected (Kennedy, 1912; Basset Smith, 1923, Giordano, 1924, Perves, 1927). The disease pre-

sented a definite problem in the Second World War, especially on the Pacific fronts (Cavanagh, 1943; Hyman, 1943; Carson, 1944; Kisner and Lisansky, 1944; Stewart, 1944; Johnson, Martin, and Breslow, 1946).

The Rôle of *Aedes aegypti* and other Mosquitoes

Graham (1903) was probably the first worker to suspect the mosquito as the vector of dengue fever, and he stated that the disease was caused by a protozoan parasite which was transmitted by *Culex fatigans* Although he appreciated the part played by the mosquito, he failed to identify the correct species and, moreover, was mistaken with regard to the existence of a protozoan parasite in certain fluids Later Bancroft (1906) in Brisbane succeeded in infecting 2 human volunteers by exposing them to the bites of infected *Aedes aegypti.* The results were sufficiently convincing, but they have been criticized by Armstrong (1923) on the grounds that dengue was prevalent in the locality in which the experiments were performed. In their classical experiments Cleland, Bradley, and McDonald (1918, 1919) excluded this fallacy by transporting *A. aegypti* from the endemic area to Sydney, which was free from the disease, and where they infected 4 volunteers by permitting them to be bitten by the insects. Subsequently, the work of Cleland and his associates was confirmed by many independent investigators, and the part played by *Aedes* in the propagation of dengue established beyond all doubt.

The next matter to receive the attention of various workers was an inquiry to determine whether dengue virus was carried by *A aegypti* alone or by other mosquitoes also. Numerous species were studied by various workers, and although the *reports are unanimous in their verdict regarding the rôle of A. aegypti* as a vector, there seems to be some doubt concerning the ability of other species to [illegible] Craig (1920), *Culex fatigans* [illegible] ns colleagues (1916) denied [illegible] r (1928), Kligler and Aschner (1928), Blanc and Caminopetros (1928, 1930 *a*, and Siler, Hall, and Hitchens (1925 *a, b*), all found that C. *fatigans* was not a vector of dengue fever. Blanc and Caminopetros (1930 *a, b*) even went so far as to suggest that in Greece and Macedonia *A aegypti* was the only mosquito which transmitted the disease The weight of data, therefore, supports the view that C. *fatigans* does not actively participate in the spread of dengue.

In Manila another mosquito, *Aedes albopictus* (Skuse), was found to be equally effective in transmitting dengue fever (Simmons, St. John, and Reynolds, 1930). In Sumatra *A albopictus* was also found to be the insect vector of the disease by Snijders, Dinger, and Schuffner (1931). These workers succeeded in transporting infected *A albopictus* from Sumatra to Amsterdam, and were there able to reproduce dengue in human volunteers by subjecting them to the bites of the insects In Formosa, Morishita (1925) has accused *Stegomyia scutellaris* Walk and also *Armigeres (Desvoidea) obturbans* Walk In New Guinea, the forest-living *Aedes stegomyia scutellaris Walk.* has been incriminated, and shown to be capable of infecting volunteers in Sydney (Mackerras, 1946) Taylor (1943) has published a useful account of the entomology of *Aedes aegypti* and *A albopictus* Usinger (1944) describes *the entomology in Honolulu Chandler and Rice (1923)* have reported positive results in experiments with *Aedes argenteus* in Texas

During the course of experiments primarily designed to ascertain which insect vector is responsible for the dissemination of dengue fever, valuable data have emerged regarding the fate of the virus in different mosquitoes Results reveal that the transmission of dengue fever from man to mosquito and from the latter to a susceptible individual is not a mechanical process For example, it has been proved that species of *Aedes* which had been fed on the blood of patients were noninfective to man on the 2nd, 3rd, 4th and 6th days after they had been fed, but from the 7th day onward the insects became capable of infecting human volunteers

(see Schule, 1928, and Blanc, 1938) The length of this "extrinsic" incubation period usually varied from 7 to 10 days and appeared to depend on the prevailing temperature, as it could be diminished by heat and increased by cold (Schule, 1928). Below 18° C. infected *Aedes* ceased to transmit the virus, and this observation of Blanc and Caminopetros (1929 *a*) is of considerable importance, since it has long been recognized that the advent of frost speedily brings outbreaks of dengue to a close (see Armstrong, 1923). Interrupted feeding has been referred to by Chandler and Rice as a possible method whereby the disease can be spread. Schule (1928), on the contrary, failed to confirm this observation and he was unable to transmit dengue by performing interrupted feeding tests with 18 mosquitoes

Simmons, St. John, and Reynolds (1930) found that it took 13 to 22 days after feeding on a patient before *A. albopictus* became infective to man. Once infected, an *Aedes* may remain infective for a considerable time, and Blanc and Caminopetros (1929 *a*) demonstrated that mosquitoes could retain their infectivity for as long as 174 days Under the circumstances, it seems possible that once a mosquito becomes infected, it may remain so for the remainder of its life It is also conceivable that the insect may only be infective at certain seasons of the year and not at others, for Blanc and Caminopetros (1929 *a*) discovered that infected *A. aegypti* were noninfective in the months of January, February, and March, but became infective from April onwards Such entomological studies are of profound practical importance to the medical epidemiologist, as they supply valuable information concerning the dissemination of dengue among a community. It also explains many of the hitherto unsolved problems regarding the sudden spread of the disease which is, of itself, noncontagious in character. Thus, Craig (1920), in discussing the work of Ashburn and Craig (1907 *a*, *b*), clearly showed how cases of dengue fever could be safely nursed in the general wards of a hospital without fear or risk of infecting adjacent patients, provided that the dengue cases were placed under cover of mosquito netting These results undoubtedly add further testimony to the theory of mosquito transmission of the disease, but they fail to explain why a large number of persons suddenly develop the disease within a comparatively short time. Under the circumstances, the only satisfactory answer to the problem would appear to lie in the possibility of a large number of mosquitoes becoming infective at once Manoussakis (1931) believes that the essential factor is the presence of a large number of infected mosquitoes. This occurs gradually over a period of time, for example, in an endemic area, at the beginning of the warm weather, a few sporadic cases of dengue follow upon the bites of infective mosquitoes More of the insects then become infective by feeding on such patients, and in turn fresh cases of dengue develop, accompanied by a corresponding rise in the population of infected mosquitoes, so that at the height of the hot season there is a large population of infected *A. aegypti*. Thereafter, the size and magnitude of the ensuing epidemic are merely governed by the number of susceptible subjects existing in the locality. According to Hanson (1936), fewer mosquitoes are necessary for the spread of dengue among the members of a community than are required for the dissemination of yellow fever. Thus, Hanson has pointed out that, whereas epidemics of dengue could occur when relatively few mosquitoes were present, he has observed the decline of

bers of *Aedes* were still on t

due to the fact that in coun

munity is complete and of lifelong duration, whilst dengue immunity only lasts from 1 to 7 years, and consequently more persons of a given population may be susceptible to dengue than yellow fever.

Efforts have been made to determine whether dengue virus can be transferred from an infected to a healthy mosquito St. John, Simmons and Reynolds (1930) state that the transference is possible under experimental conditions. It was also shown that the virus could be transferred from one mosquito to another by copula-

sented a definite problem in the Second World War, especially on the Pacific fronts (Cavanagh, 1943, Hyman, 1943, Carson, 1944, Kisner and Lisansky, 1944, Stewart, 1944; Johnson, Martin, and Breslow, 1946).

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In the northern sector the disease has been noted extensively in Texas. cases have been reported from Fort Worth (King, 1907, and Graves, 1908); from Brownsville (Goldberger and McCoy, 1907); and from Galveston (Rice, 1922, 1923). In Florida, epidemics have been mentioned as occurring in Jacksonville (Porter, 1894), in Miami (Griffitts and Hanson, 1936), and in Key West (Porter, 1894). The disease has been reported in Louisiana by Scott (1923), in the Isthmus of Panama by Sutton (1904), and Carpenter and Sutton (1905), in Dutch Guiana by Bonne (1918), and in Bogota, Colombia, by Tascon (1924). Many of the large and smaller islands off the American coast have also been involved and reports of epidemics are recorded in Bermuda by Meagher (1916), in Cuba by Allen (1908), and Guiteras and Cartaya (1906); in Puerto Rico the 1915 epidemic was described by King (1917), and a smaller outbreak involving 100 persons in St. Thomas island is mentioned by Lane (1918).

South of the equator, cases have been reported from Ecuador by Carbo-Noboa (1926), and in the Argentine by Kraus (1916), who states that the disease was probably imported directly from Spain.

Australasia.

Outbreaks of the disease have occurred in New South Wales in the years 1898, 1906, 1916, and 1926 (see Ferguson, 1926, Goldsmid and Crosse, 1916, and Goldsmid, 1917). North Queensland was affected in 1897 (Hare, 1898), and Brisbane in 1905 (Robertson *et al.*, 1905). Subsequently cases were described in this area by O'Brien (1908), Scott (1911), Davidson (1911), and more recently an outbreak was recorded by McCallum and Dwyer (1927). An epidemic in 1927–8 at Port Darwin has been recorded by Murray (1931). Later papers are those of Walker *et al.* (1942), Lumley (1943), Zussman (1944). It occurs in New Guinea (Mackerras, 1946)

Pacific Islands.

Numerous cases have been recorded from time to time as occurring in this part of the globe In the Philippines they have been reported by Bell (1906) and Jones (1909), in New Caledonia by Nicolas (1927), and in Nouméa (New Caledonia) by Cozanet (1910). In the Fiji Islands a large epidemic was observed in 1885 by Skottowe (1890), and a later recrudescence in 1907 by Montague (1908). In American Samoa, in 1930, an epidemic occurred during which 2,842 cases developed within 2 months (Hargrave, 1931). The Hawaiian Islands were affected by dengue in 1903, and Wilson (1904) has published an account of this outbreak. From 1912 onward the disease was rare, but there was a sharp outbreak in 1943, terminated by vigorous anti-Aedes measures (Wilbar, 1947).

Perry (1948) concludes that *A aegypti* is the main vector in the S Pacific.

Greece, Turkey, Palestine, Syria, and neighboring country.

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tion. Simmons *et al.* (1931) suggested, however, that although this was possible experimentally it was unlikely that the virus could be transmitted from insect to insect in this manner in nature. Under artificial conditions, St. John, Simmons, and Reynolds (1930) were able to infect healthy *Aedes* by inducing them to feed through guinea-pig's skin on a mixture of blood and dengue virus. Such artificially infected mosquitoes were then proved to transmit the virus when they bit volunteers.

The distribution of virus in the body of infected *A. aegypti* has been investigated by Holt and Kintner (1931). These workers allowed laboratory-bred mosquitoes to feed on cases of dengue fever, and 20 days later they dissected the insects, and injected the various organs into human volunteers. From this work, the virus was found to be present in the salivary glands, ovaries, testes, stomach, intestines, and the legs of infected mosquitoes, and volunteers who had been inoculated with each of these parts developed dengue fever in due course. Dengue virus, therefore, appears to be evenly distributed throughout the tissues of the mosquito, a finding which has led Holt and Kintner to the conclusion that during the sojourn of the virus in the body of the vector it undergoes simple multiplication, and probably does not undergo cyclical development. Further support of this view is supplied by the fact that the virus can be serially propagated through at least 4 human passages without any apparent attenuation in virulence (Cleland, Bradley, and McDonald, 1919). Neither did alternate passage between man and mosquito for 6 generations appear to influence its pathogenicity (Siler, Hall and Hitchens, 1926).

In summarizing the literature on the mosquito-transmission of dengue virus, it seems to be universally accepted that under normal circumstances *Aedes aegypti* is the commonest natural vector of the disease, and once infected the insect may so remain for the remainder of its life. On the other hand, the virus does not seem to survive long in certain mosquitoes, such as *Culex fatigans*, since Simmons, St John, and Reynolds (1931) report that this mosquito digests and kills the virus in 14 days. It would be unwise, however, to assume that *C. fatigans* or, indeed, any blood-sucking insect is totally incapable of transmitting dengue because it may well do so, merely by the mechanical transference of blood during interrupted feeding (Simmons, St. John, and Reynolds, 1931). The positive result reported by Craig (1920) may have been due to this cause, as the culex which was induced to bite a volunteer had fed upon a patient only some 2 days previously. The virus may have been carried by the mosquito for this short time. Likewise, *A. aegypti* may propagate infection by mechanical inoculation of infected blood as well as by the natural medium of infective saliva.

Lumley (1943) summarizing the experimental work, mostly alluded to above, concludes that patients can infect *Aedes* from 1 day before to 5 days after the onset of illness, the average "extrinsic" incubation in the mosquito is 8 days, and once infective it may remain so for life, Aedes probably do not infect their eggs

Control of Dengue

The control is largely a question of antimosquito measures, such as clearing away unnecessary water containers, oiling, and spraying, patients should be nursed under nets (Walker *et al.*, 1942, Gilbertson, 1945). DDT sprays are recommended

Geographical Distribution of Dengue Fever

Armstrong (1923) lists the main epidemics in various parts of the world. The disease is most prevalent in the following countries:

America.

The distribution of cases tends to be confined to a belt extending from 36° north to 35° south of the equator

In the northern sector the disease has been noted extensively in Texas: cases have been reported from Fort Worth (King, 1907, and Graves, 1908), from Brownsville (Goldberger and McCoy, 1907), and from Galveston (Rice, 1922, 1923). In Florida, epidemics have been mentioned as occurring in Jacksonville (Porter, 1894), in Miami (Griffitts and Hanson, 1936), and in Key West (Porter, 1894). The disease has been reported in Louisiana by Scott (1923), in the Isthmus of Panama by Sutton (1904), and Carpenter and Sutton (1905); in Dutch Guiana by Bonne (1918); and in Bogota, Colombia, by Tascon (1924). Many of the large and smaller islands off the American coast have also been involved and reports of epidemics are recorded in Bermuda by Meagher (1916), in Cuba by Allen (1908), and Guiteras and Cartaya (1906); in Puerto Rico the 1915 epidemic was described by King (1917); and a smaller outbreak involving 100 persons in St Thomas island is mentioned by Lane (1918).

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Greece, Turkey, Palestine, Syria, and neighboring country.

These areas may be regarded as the home of the disease, and Blanc and Caminopetros (1930 *a*) have given a good account of the clinical, epidemiological, and experimental aspects of dengue fever as met with in these parts, and their paper contains a map showing the distribution of *Aedes* in Greece The reports contained in medical literature describing outbreaks of dengue fever in these regions are too numerous to be cited individually, and it is, therefore, only possible to refer the reader to a few

At Athens and in Greece during 1927–8 about 20,000 are reported to have suffered (Copanaris, 1928, and Tsiminakis, 1930) In Constantinople cases have been reported by Siotis (1889), Maléas (1889), Spadaro (1890–1), Zohrab (1890), and Stefko (1917), who has recorded the 1916 epidemic in Turkey. In the Dardanelles and Macedonia it was reported by Armand-Delille (1916), in Rhodes by Poggi (1929), in Cyprus by Carageorgiadès (1889), in Naples by Cantani (1890), and

in other areas by Adrien (1918). The Smyrna epidemic of 1889 is recorded by Crediropoulos (1890), Diamantopulos (1890), and Stamatiades *et al.* (1890). About the same time Wortabet (1887) and De Brun (1889) noted it at Beirut in Syria. The disease has been described in Baghdad, Iraq (Fleming and French, 1947). Couvy (1921) described later epidemics in Beirut and Hitti and Khairallah (1946). In Palestine Masterman (1913) gives an account of the disease, and Dive (1927) noted it at Aden in Arabia.

Europe.

Apart from the above-mentioned areas of the continent in which dengue has been observed, cases have been reported at Lisbon by Tavares (1889), and in Vienna by Eugling (1922). The latter stated that the small outbreak which occurred in the summer of 1921 in Vienna was not due to infection acquired locally, but was introduced by Macedonian immigrants.

Africa.

The disease is widely distributed throughout this continent and cases have been reported in Egypt by Sandwith (1888), Phillips (1906), and Kamal (1928); in the Sudan by Balfour (1907), Saigh (1906), and Archibald (1917); in Zanzibar by Godding (1890), in East Africa by de Sousa (1924) and McCarthy and Brent (1943), and at Accra in the Gold Coast by Corson (1921). Cases have also been reported to occur among the French naval forces stationed at Dakar (see Bideau, 1925, Perves, 1927, 1928).

India.

Dengue is found throughout the country and cases have been mentioned as occurring in Sialkot by Fooks (1908), in Calcutta by Kennedy (1912), in Meerut by Khan (1913), in Guzerat by Dalsukhram (1913), the Punjab by Farrer (1924). Other accounts are to be found in the writings of Rogers (1909), Megaw (1919), and Barrand (1928).

In Ceylon the disease is also relatively common, and cases have been recorded by Spaar (1925).

The Far East.

Dengue has an extensive distribution throughout the Far East of the globe. Cases have been reported in Burma by Pridmore (1902), and in Rangoon by Jafar and Singh (1932), in Penang by Skae (1902), in Indo-China by Vassal and Brochet (1909), in Cochin-China by Lalluyaux (1914), who gives a description of the 1873 outbreak in that country, and Millous (1929), who records another outbreak involving 1,000 cases in Cochin-China in 1927. In Tonkin (Annam) the disease has been observed by Gaide (1913), in the Dutch East Indies by De Langen (1925), and in Hong Kong by Basset-Smith (1923). Farther east, Hanabusa (1917) noted it in Formosa, Goto (1923) in Pescadores, and Miki (1910) in Tokyo. The epidemic of 1922 in Japan has been reported by Kaku (1923).

THE 1934 INTERNATIONAL CONVENTION FOR MUTUAL PROTECTION AGAINST DENGUE FEVER (1935)

Public health legislation designed to control the spread of dengue fever was introduced at a congress convened for this purpose at Athens on July 25 1934. Appropriate legislation was agreed upon by the respective governments of Albania, Germany, Great Britain, Bulgaria, Egypt, Spain, France, Greece, Italy, Roumania, Turkey, Soviet Russia, and Yugoslavia, and the resolutions carried have been incorporated in a list of 9 articles. The medical measures recommended are as follows

Article 2.

When in any of the territories to which the present Convention applies, an epidemic of dengue fever shall be discovered in a port or in a district adjoining a port, the sanitary authority of the said port shall recommend captains of vessels and ships' doctors, if such are on board, immediately after leaving the port, to search out and destroy mosquitoes and their larvae in all the accessible parts of the ship, especially in the cabins, crew's quarters, store rooms, kitchens, stoke holes, water containers, and all places likely to harbor mosquitoes

The sanitary authority shall request the doctor, or failing him the captain, to take such measures, as may be necessary to insure that if cases of dengue fever occur on board the patients may be isolated under conditions such that they cannot be bitten by mosquitoes.

Article 3.

In any of the territories to which this Convention applies, all ships, coming from a port in which there is an epidemic of dengue fever and arriving in a port whose sanitary authority has reason to fear that the disease may spread owing to the presence of a large number of mosquitoes capable of transmitting it, may be subjected to the following measures —

(*a*) Boarding —The doctor, or failing him the captain, shall give a reply to the question "Are there, or have there been, on board any persons suffering from dengue fever?"

(*b*) Medical Inspection —Patients who have developed dengue fever within less than 5 days and desire to leave the ship shall be landed by day and shall be isolated on land, in accordance with the instructions of the competent sanitary authority, under conditions which shelter them from mosquito bites, until a period of 5 days has elapsed since the illness began

(*c*) Inspection of the ship in order to ascertain that there are no stegomyia on board, taking into account any measures already taken on the voyage If any stegomyia are found on board the sanitary authority may have the mosquitoes destroyed

(*d*) In exceptional cases, the sanitary authority of the port may, if he considers that circumstances render it necessary, place under surveillance those passengers who have disembarked and confine the crew on board except for purposes of duty, until a period of 8 days has elapsed since they were exposed to risk

Article 4.

When an epidemic of dengue fever has broken out in one of the territories to which the present Convention applies, and which has a land frontier bordering on another territory to which the Convention also applies, the sanitary authorities of the latter territory may, if they consider it necessary, take the following measures at the frontier

(*a*) Passengers may be placed under surveillance for a period not exceeding 8 days from the day of exposure to the infection

(*b*) Passengers suspected of having developed dengue fever within less than 5 days may be isolated according to the instructions of the sanitary authorities in conditions in which they are protected from mosquito bites until a period of 5 days has elapsed from the beginning of the illness

CLINICAL FINDINGS

The Incubation Period

The incubation of the naturally acquired infection is usually 5–8 days, but may vary from 3–15 (Ashburn and Craig, 1907 *a*, *b*, Armstrong, 1923, Cleland, 1930, Lumley, 1943)

In the case of human volunteers experimentally infected by mosquitoes, the shortest period was found to be 3 to 4 days (Ashburn and Craig, 1907 *a*, *b*), and the longest 9 to 10 days (Cleland, Bradley, and McDonald, 1919) Much the same results were observed with regard to subjects inoculated with infected blood, some of whom developed the disease in 2½ to 3 days (Ashburn and Craig, 1907 *a*, *b*), and others in 9 to 10 days (Cleland, Bradley, and McDonald, 1918, 1919). Blanc and Caminopetros (1930 *b*) found that the incubation was 3 to 7 days in the case of the experimentally induced condition.

in other areas by Adrien (1918). The Smyrna epidemic of 1889 is recorded by Credtropoulos (1890), Diamantopulos (1890), and Stamatiades *et al.* (1890). About the same time Wortabet (1887) and De Brun (1889) noted it at Beirut in Syria. The disease has been described in Baghdad, Iraq (Fleming and French, 1947). Couvy (1921) described later epidemics in Beirut and Hitti and Khairallah (1946). In Palestine Masterman (1913) gives an account of the disease; and Dive (1927) noted it at Aden in Arabia.

Europe.

Apart from the above-mentioned areas of the continent in which dengue has been observed, cases have been reported at Lisbon by Tavares (1889), and in Vienna by Eugling (1922). The latter stated that the small outbreak which occurred in the summer of 1921 in Vienna was not due to infection acquired locally, but was introduced by Macedonian immigrants.

Africa.

The disease is widely distributed throughout this continent and cases have been reported in Egypt by Sandwith (1888), Phillips (1906), and Kamal (1928), in the Sudan by Balfour (1907), Saigh (1906), and Archibald (1917), in Zanzibar by Godding (1890), in East Africa by de Sousa (1924) and McCarthy and Brent (1943); and at Accra in the Gold Coast by Corson (1921). Cases have also been reported to occur among the French naval forces stationed at Dakar (see Bideau, 1925, Perves, 1927, 1928).

India.

Dengue is found throughout the country and cases have been mentioned as occurring in Sialkot by Fooks (1908), in Calcutta by Kennedy (1912); in Meerut by Khan (1913), in Guzerat by Dalsukhram (1913), the Punjab by Farrer (1924). Other accounts are to be found in the writings of Rogers (1909), Megaw (1919), and Barrand (1928).

In Ceylon the disease is also relatively common, and cases have been recorded by Spaar (1925).

The Far East.

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3rd or 4th day. Subsequently, recovery may take place in an uninterrupted manner, or else a second bout of pyrexia may occur with a further slight rise of temperature The latter is seldom as high as that of the first, and a double rise of temperature when present is referred to graphically as producing the "saddle back" type of chart (see Fig 31, and also Doerr and Russ, 1914). Antipyretics appear to

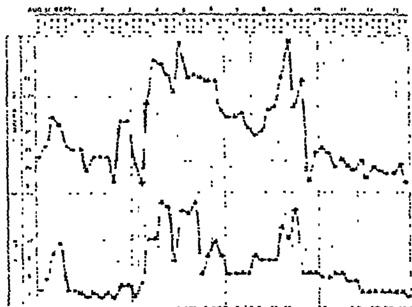


FIG. 31 Temperature and pulse chart in a case of experimentally induced dengue fever studied by Ashburn and Craig (1907)

(Reproduced from the *J infect Dis*, 1907, *4*, 440)

have little effect in combating the pyrexia (Sutton, 1904), and each occasion on which the temperature falls is accompanied by profuse sweating.

Lumley (1943) describes 5 types of temperature chart (1) continued fever, (2) saddleback type, (3) diphasic, with a normal temperature on the 3rd to 4th day, (4) monophasic type, with pyrexia for 1-4 days, (5) mild or afebrile types (see also Ross, 1948)

The Urine

albu

sugg

The Blood Picture

A well-marked leukopenia principally affecting the polymorphonuclear cells is a diagnostic feature (Balfour, 1907; Stitt, 1907, see also Ashburn and Craig, 1907 *a*, *b*, Vedder, 1907). A shift to the left of the Arneth count has been regarded by Simmons, St John, and Reynolds (1931) as most reliable (see also Hughes, 1946) Abnormal lymphocytes or monocytes with a vacuolated cytoplasm and coarse granular inclusions have been reported (Kisner and Lisansky, 1944, LeRoy and Lindberg, 1944). A marked neutropenia and lymphocytosis may persist into convalescence, along with the presence of Turk cells

The possible existence of a leukotoxin in the circulating blood during an attack of dengue fever has been suggested by Joannidès (1930).

Course of the Disease

The onset may be sudden without any indications of prodromal symptoms. Alternatively the disease may be ushered in with a feeling of chilliness, general malaise, headache, loss of appetite, and a dry throat. These are followed by the development of pains in the back and other parts of the body. A slight primary rash is sometimes seen during the first day or two of the disease, and usually affects the face, chest, neck, arms, the knees or elbows The face is often flushed, the skin is red, hot, and puffy, and the conjunctivae and mucous membranes become injected. The primary erythema, if present, may either disappear or else merge into the secondary rash which develops later in the disease. The latter commences with the second rise of fever about the 4th or 5th day of the illness, first making its appearance at the bases of the thumbs, and later extending over the hands, feet, and forearms In character it may be either morbilliform or scarlatiniform in type, and may on occasions be indistinguishable from the rash of measles It lasts for 3 to 4 days, causes itching of the skin covering the palms and soles, and *during convalescence there may be desquamation.*

The aches and pains which occur in various parts of the body during an attack of dengue fever are highly characteristic of the disease. These are, headache, backache, severe pains in joints, muscles and their tendinous insertions, and behind the eyes. Sometimes there is considerable discomfort on attempted movement of the ocular muscles and tenderness on palpation of the eyeballs. Pain in the testicles and groins may also be present. Glandular enlargements have been observed in certain epidemics only, for example Goldberger and McCoy (1907) noted them in the Brownsville (Texas) epidemic, Levy (1920) in Galveston, and Pridmore (1902) noted extensive enlargement of cervical, axillary, inguinal, and supracondylar glands in the Burma (1902) outbreak. Likewise Lane (1918) and Castellani (1917, 1938) have drawn attention to their presence. Splenic enlargement is sometimes present during pyrexia (McMullin, 1919).

Dengue in Children

At one time it was thought that children were more resistant to the disease than adults, but recent reports by various observers have shown that children ranging in age from 3 months to 15 years appear to be equally susceptible (see Choremis, 1930, and Vera, 1929) In the 1928 Athens epidemic Spyropoulos (1931) observed that among children vomiting and convulsions were common, the initial rash was well marked on the face, and the pains in joints were more frequently seen in the older children than in the younger ones. Hemorrhagic symptoms occurred less readily than in adults, and on the whole the mortality was low

Influence of Race

There appears to be no racial immunity, but some reports suggest that white persons are more susceptible than colored to dengue infection (see Christie, 1872, and Simmons, St John, and Reynolds, 1931)

The Pulse

Slowing of the pulse becomes noticeable about the 3rd day of illness and the rate becomes progressively diminished, so that during the second bout of pyrexia it may be as low as 45 beats per minute (Stitt, 1913). Hyman (1943) finds the cardiovascular signs to be due to excessive vagal or autonomic response (see also Portocalis and Flora, 1928).

The Temperature

This rises rapidly to about 102° to 103° F, and may rarely be 105° F or more, it thereafter gradually decreases during the first 2 days to reach normal about the

For a time the etiological significance of leptospira as a possible causal factor of dengue fever ground owing to the clinical re nd more especially because at from alleged cases of yellow fever. For example, Craig's (1920) judgment was greatly influenced by Noguchi's findings and he, not unreasonably, assumed that both diseases possessed a common etiological factor. Subsequent work, however, on the cause of yellow fever revealed that this disease was due to a filtrable virus, and in all probability the material supplied to Noguchi by clinicians in South America originated from unrecognized cases of infectious jaundice or Weil's disease (see p. 459). Subsequently, the leptospira theory of dengue fever was reinvestigated by Siler, Hall, and Hitchens (1925 *a*, *b*, 1926) in the Philippines, by Chandler and Rice (1923) in Texas, and by Kligler (1928) and Kligler and Aschner (1928) in Palestine, all of whom reported negative results Thus, the Palestine workers could not detect leptospira in the blood of 9 cases of dengue, either by cultural or direct microscopical methods of examination. They, moreover, were unable to find leptospira in several thousands of insects which had been caught in infected houses. Finally, it was proved that a leptospira is unable to survive for more than 16 hours in *Stegomyia* and no longer than 48 hours in *Phlebotomus papatasii*. Results such as these offered conclusive evidence in favor of the fact that dengue fever is not a spirochetal infection. Similar observations regarding the possible leptospira theory of sandfly fever were carried out simultaneously by Kligler and Aschner (1928), and these are described in Ch XLIV.

EXPERIMENTAL WORK ON THE VIRUS

Human Transmission and Filtrability

Modern research on the virus agent of dengue fever originates from the work of Ashburn and Craig (1907 *a*, *b*) who studied the condition at Fort William McKinley, Rizal, in the Philippine Islands during an epidemic that affected over 800 soldiers One hundred and twenty eight cases were transferred to the hospital at Manila (in which city the disease was absent at the time) and experimental work on human volunteers was performed there. First, the blood of numerous patients was examined for the possible presence of organisms by direct microscopy and cultural methods, but the results were negative throughout. These two workers then showed that, when blood was removed from cases of dengue fever on the 3rd or 4th day of the illness and inoculated into 11 volunteers, it reproduced dengue in 7 of them. The longest incubation period was found to be 7 days, the shortest 2½, and the average 4 days

Experiments were next conducted in order to find out whether the infective agent was filtrable Accordingly, blood was obtained from patients, rapidly defibrinated, diluted in saline, passed through a Lilliput diatomaceous earth filter candle (which was c
intravenously with
cal attack of dengue
ceptbile individual is
mura, 1917) Further researches of a similar character were performed in Australia by Cleland, Bradley, and McDonald (1919) who passed the virus through the Pasteur-Chamberland F filter They, moreover, discovered that it was uniformly distributed throughout whole blood, being present in filtrates of serum or citrated plasma Likewise, well-washed corpuscles proved to be infective. The virus was

Cerebrospinal Fluid

Manoussakis (1928) demonstrated that this fluid contains the virus and is infective. There may be a slight increase in cells and albumin (LeGac and Servant, 1939).

COMPLICATIONS

Complete recovery is usual, but on rare occasions complications supervene.

Hemorrhages. In severe cases these may occur from the stomach, nose, genitourinary tract, kidneys, and bladder. Subcutaneous skin hemorrhages are also prone to occur, and Rumpel Leede's and Marwitz-Deriecke's tests become positive (Georgopoulos, 1928).

Eye lesions may take the form of paralysis of various muscles, or iritis and sometimes even keratitis. Richardson (1927) reports 6 cases of keratitis which he observed during convalescence in the Jacksonville epidemic. Dengue fever has also been known to precipitate an attack of acute glaucoma in an individual.

The central nervous system Dengue fever may be accompanied by signs and symptoms of disease referable to the nervous system. Perhaps the commonest of these take the form of attacks of mental depression during illness, which may occasionally be prolonged into convalescence Severer involvement of the central nervous system, e g. with paralysis, may occur (Tsiminakis, 1930, Avaritsiotis, 1930, Kaplan and Lindgren, 1945). It seems possible that a true dengue encephalitis may occur (Lumley, 1943)

Instances in which *orchitis* or *oophoritis* have followed an attack of dengue have been noted (Nicolas, 1927, Weyrauch and Gass, 1946). *Dengue fever as a pyrotherapeutic remedy* has been recommended by Pamboukis (1930) for the treatment of certain nervous diseases such as parkinsonism following encephalitis, epilepsy, and general paralysis of the insane. *Surgical complications* arising during the course of dengue infection have been described by Kondoleon and Joannidès (1930).

POSTMORTEM FINDINGS

The mortality due to dengue is exceedingly low and, for example, McCallum and Dwyer (1927) estimated that it was about 0 03 per cent. during the Queensland epidemic In consequence of the small death rate, little is known regarding the morbid anatomical or histological features of the severest cases Photakis (1929) reports that in autopsies performed by him on fatal cases, he found enlargement of the liver and degeneration of the myocardium The kidneys and suprarenal glands appeared to be normal No renal lesions could be found to account for hematuria which occurred prior to death in certain cases

ETIOLOGY

In earlier studies numerous workers at different times have claimed to have discovered the specific etiological agent of the disease. Thus, various types of bacterial and protozoal parasites have been found in the blood of patients by McLaughlin (1886), Eberle (1904), Reiche (1906), Nagib (1910), and Graham (1903) Holt (1923) observed a pleomorphic bacterium in human blood as well as in that of "infected" animals, and Harris and Duval (1924) isolated globoid bodies in cultures made from patients McMullin (1919) suggested that the disease was an allergic manifestation brought about by repeated introductions of mosquito protein

The Spirochete Theory

Spirochetes were observed in the sputa of certain human cases by Allen (1908) They were also isolated from blood by many investigators such as Couvy (1921), working at Beirut in Syria, and also by Vervoort (1922) and van der Velde (1923) in the Dutch East Indies

quinine. The virus cannot be absorbed from serum by treatment with calcium carbonate or tapioca (see Blanc and Caminopetros, 1930 *b*, *c*)

Animal Inoculation Experiments

There is no evidence that either domestic [illegible] in nature (Cleland, 1930). Attempts to infe[illegible] and rabbits proved negative (Simmons, St. [illegible] Schlesinger (1945), however, adapted virus to mice by the cerebral route, and produced a modified attack in man with passage virus.

Only partial success has been achieved in the case of larger animals such as guinea-pigs and monkeys Inoculation of the latter causes a mild febrile reaction which may sometimes be accompanied by leukopenia. During this period of transient illness the virus can often be demonstrated in the animal by inoculating a human volunteer with a small quantity of its blood. Animal infection of this character has been referred to by Nicolle (1928) as "inapparent" infection because, although the virus survives and multiplies for a time in the animal, it fails to establish itself in it Thus Blanc, Caminopetros, and Manoussakis (1928), showed that 5 days after inoculation of a guinea-pig with infected human blood, the animal's blood proved pathogenic to a human volunteer. Likewise Findlay (1932) found that the same held true in the case of a rhesus monkey whose blood contained the virus 7 days after it had been infected, although the animal only showed a slight leukopenia without obvious signs of illness. Blanc, Caminopetros, Dumas, and Saenz (1929) made a similar observation, when they experimented with the following monkeys *Cynomolgus sinicus*, *C. fascicularis*, *Cercopithecus callitrichus*, *Cercocebus aethiopis*, and *Papio babuin*. They found that the blood of the animals first became infective for humans 5 to 8 days after inoculation, but ceased to be so after the 12th day. Attempts to transmit dengue virus by passage from one monkey to another were unsuccessful. Simmons, St John, and Reynolds (1931) found that the Japanese monkey, *M fuscatus*, was also susceptible and exhibited a transient febrile illness following inoculation Simmons (1931) obtained positive results with *M. philippinensis* as well as in *C fascicularis*, *C callitrichus*, and *M. fuscatus* monkeys He found that the animals suffered from latent dengue infection and, although their blood was noninfective for humans 24 hours after injection, it acquired infectivity from the 5th to the 8th day. On the 12th day, however, the virus disappeared from their blood, and thereafter the monkeys became solidly immune to reinfection for a period of 15 days In view of the ability of monkeys to harbor dengue virus in their blood stream, Simmons (1931) has suggested that lower animals may act as reservoirs of infection for dissemination of the disease in certain areas

IMMUNITY REACTIONS IN MAN

A number of workers has found that active immunity persists for a year or more on recovery from infection (e g, Blanc and Caminopetros, 1929 *b*, Sharp and Hollar, 1935) Lumley (1943) concludes that one attack tends to give a short-lived immunity, but that subsequent attacks tend to be milder, residents in endemic areas develop, by constant exposure, some resistance to attack

Attempts at active immunization have been made. Simmons, St. John, and Reynolds (1931) had negative results with vaccines prepared from infected mosquitoes and human blood St John and Holt (1931) used infected monkey tissue. Blanc and Caminopetros (1930 *b*) achieved some success with bile-treated human serum More success may follow the use of mouse-brain virus (Sabin and Schlesinger, 1945)

L_7 and L_{11} bougies, and Kligler and Aschner (1928) filtered it through the Berkefeld N candle. Simmons, St. John, and Reynolds (1931) showed that it could pass through the Berkefeld V filter.

The infectivity of blood from human volunteers has been confirmed by subsequent investigators. The virus will survive for some time and specimens can be sent to distant laboratories (Philip, 1946). Lumley (1943) summarizes the position by stating that virus is present in the blood as a rule from 1 day before to 5 days after the onset of illness.

Morphology

Since the virus is very small in size, it lies beyond the range of visibility of the ordinary microscope, and consequently it has not yet been possible to demonstrate it in stained preparations Sellards and Siler (1928) observed, however, that large numbers of rickettsia-like organisms could be found in dengue-infected *Aedes* mosquitoes, in microscopic preparations stained by Giemsa or Mallory's eosin methylene blue technique. Such rickettsiae were absent from control mosquitoes, but in these there was present instead a protozoan parasite, *Lankesteria culicis*, due to which a certain amount of confusion arose. The results of the investigation are thus inconclusive. Coles (1937) has described minute granules in infected blood which he has called *Maculae dengui*

Cultivation

Shortt, Rao, and Swaminath (1936) inoculated developing chicken embryos (see Ch. XIII) with the blood of dengue fever cases derived from patients on the first day of their illness. Eggs were inoculated with either plasma or serum from 19 typical cases of dengue fever, and of these 15 produced lesions on the surface of the chorio-allantoic membrane In appearance these were similar to changes reported in their experiments on sandfly fever (see p. 523) but with certain differences. For example, the lesion of dengue virus on the membrane was considerably thicker than that caused by sandfly fever virus, and whereas on section the edges of the former were approximately parallel to each other, the margins of the latter tended to be curved and bulging in their contour. Microscopically also certain differences have been detected The distribution of inclusion material present in dengue virus lesions of the membrane appeared to be identical with those reported in the case of sandfly fever virus.

Reaction to Physical and Chemical Agents

Cold The virus retains its infectivity in human blood if stored in a refrigerator at 0° C. for about 2 months (Blanc, Caminopetros, and Manoussakis, 1928).

Heat. Manoussakis (1928) found that the thermal death point of the virus was 50° C. for half an hour, and that exposure to bright *sunlight* for 30 minutes failed to inactivate it.

Desiccation The infectivity of human serum can be preserved for long periods of time by drying it Such material has been employed by Findlay (1932) for the inoculation of human volunteers in London Simmons, St John, and Reynolds, (1931) found that the virus survived for a longer time in living than in dead *Aedes,* in which it only existed for about 4 days at 18° C.

X-rays. The action of these rays on *Aedes* infected with dengue virus has been investigated by Holt, Fleming, and Kintner (1931), who found that exposure of the insects to erythema doses neither killed the virus, nor modified the course of the disease in human volunteers who were permitted to be bitten by the mosquitoes after these had been treated with the rays *Ultraviolet light* likewise failed to exert any effect on the virus

Chemicals The virus resists a 1 in 800 dilution of formalin for 5 hours, 0 5 per cent. cholesterin for 15 minutes, 1 in 500 neutral red for 7 hours, 95 per cent al-

DENGUE FEVER

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Although resistance following the disease may be lasting, the serum of an immune subject appears to contain no virus neutralizing antibodies Many workers have drawn attention to this point. For example, Blanc and Caminopetros (1929 *c*) showed that neither whole blood nor serum derived from convalescent cases possessed any protective properties. Holt, Fleming, and Kintner (1931) also obtained negative results with convalescent sera. Partial success has been obtained with complement fixation tests using ground-up infected mosquitoes as antigen

Efforts to prepare therapeutic or prophylactic antisera have failed, Blanc, Caminopetros, and Giroud (1929) found that antisera prepared in rabbits were useless. Yellow fever antiserum and anti-hog cholera serum did not exert a beneficial effect on cases of dengue fever (see also Dinger and Snijders, 1931).

RELATIONSHIP TO OTHER VIRUSES

Recently, numerous tests have been performed in order to elucidate whether dengue virus possesses any properties in common with yellow fever virus on the one hand and Rift Valley fever virus on the other. Dinger and Snijders (1931) made the important observation that monkeys which had recovered from an attack of dengue fever were subsequently resistant to infection with yellow fever virus But although this was the case, the sera of dengue-immune monkeys failed to neutralize yellow fever virus. Hanson (1936) mentions an interesting case in which an individual who had suffered from dengue fever developed an attack of yellow fever a year later. Blanc, Caminopetros, and Giroud (1929) found that yellow fever antiserum did not neutralize dengue virus, and Sharp and Hollar (1935) reported that human serum from a case of dengue fever gave a negative mouse protection test against yellow fever virus. In cases where doubt exists, the clinical differential diagnosis between dengue and yellow fever may thus be established by application of the mouse protection test (see p 507) With regard to the possible relationship of dengue virus to Rift Valley fever virus, Findlay (1932) reported that monkeys which had been immunized against dengue virus were not resistant to Rift Valley fever virus, and vice versa This subject is discussed more fully in Ch. XXXVI

Challenge experiments in human volunteers did not elicit any relationship between dengue and Colorado tick fever (Florio *et al*, 1946, Pollard *et al*, 1946, Koprowski and Cox, 1947), or Bullis fever (Livesay *et al*, 1946)

FORT BRAGG (PRETIBIAL) FEVER

In the summer of 1942, a dengue-like illness attacked troops at Fort Bragg, North Carolina, there were similar outbreaks in the summer of 1943 and 1944 Tatlock (1947) describes the clinical features of a case as follows. sudden onset of headache, with fever, and generalized aches, 5 days after onset he developed a maculopapular eruption over the pretibial areas, the fever lasted for 5 days, and the patient made a rapid recovery The causal agent was found in the blood 48 hours before and after the onset of fever Tatlock isolated an agent, presumably a virus, by inoculating guinea-pigs with blood from a human case The agent induced fever in guinea-pigs and rabbits, and killed hamsters The agent was passed in animals, or fertile eggs (by the intravenous route), and after prolonged passage infected human volunteers Some of these developed fever for 1–3 days, and showed the clinical picture of Fort Bragg fever

The causal agent appeared unrelated to LCM, Q fever, Rocky Mountain spotted fever, sandfly and dengue fevers

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YELLOW FEVER—HISTORICAL ACCOUNT

Place of Origin

... of yellow fever is to be ... has endeavored to eluci- ... epidemiological records. ... in West Africa and was introduced into America by sailing vessels and their personnel The insect vector *Aedes aegypti* was in all probability also imported into the New World at the same time. In his efforts to trace the chronological sequence of the earliest recorded outbreaks of yellow fever, Carter's researches have been hampered by the fact that, whereas the Spanish-American literature was plentiful and complete, no native documents existed relating to the incidence of the disease in West Africa

According to Carter, the first reliable account of yellow fever in America was given in 1648, but Hirsch (1883) mentions that Du Tertre of Guadeloupe referred to it in the year 1635. In Africa it was not described until 1778, when Schotte (1782) drew attention to its presence in Senegal territory. Although yellow fever was recorded in America about 130 years prior to its recognition in Africa, there is evidence to suggest that it originated in Africa, and then spread westward. For example, when yellow fever was noticed in the Yucatan in 1648, it was clearly recognized in that land as a new and hitherto unidentified disease, but Schotte's account of it in Senegal in 1778 hints that the malady was merely a recrudescence of a similar condition encountered previously.

America.

According to Carter, yellow fever was not present in America either at the time of the landing of Columbus or for many years thereafter. Following its appearance in the Yucatan in 1648, Carlos Juan Finlay (1912) reported that it occurred in 1649 in Cuba. Here the disease affected the fleet of Don Juan Pujadas as it lay at anchor off Havana. After 1665, yellow fever disappeared from Cuba until 1762, when it was reintroduced by prisoners sent from Vera Cruz to assist in rebuilding Cabanas after its siege and capture by Pocock and Albemarle. Subsequently the disease remained endemic in the island up to 1901, when Gorgas is reported to have instituted antimosquito measures and thereby eradicated it. Carter points out that Cuba was the first place from which yellow fever was abolished by the introduction of mosquito control. In 1640 Pernambuco was affected, in 1665 the island of St Lucia was invaded, and by 1658 the infection had spread throughout Brazil Thereafter it was spread through Mexico and Central America by the movements of troops engaged in wars waged at the time.

Colombia.

In Colombia, the emerald mines at Muzo were first attacked in 1885, allegedly owing to the introduction of infection by troops from Honda The Muzo mines have long remained a permanent endemic source of infection and Gorgas (1917) investigated epidemics in 1916 and 1923 without discovering cases of yellow fever or *Aedes aegypti* all of which, in the light of current knowledge, are suggestive of jungle yellow fever. Cases occurred at Bucaramanga in 1810 and 1923, Buenaventura on the Pacific coast in 1920, and after a period of 6 years of freedom from infection in Colombia, the town of Socorro Santander suffered in 1929 (Smith, Bevier, and Bugher, 1943)

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was conveyed from there into Granada in 1793 by the ship Hankey (see Chisholm, 1801).

THE PART PLAYED BY SAILING VESSELS IN THE SPREAD OF YELLOW FEVER

Ships have long been recognized as an agency by which the disease has been disseminated, and in 1883 Hirsch categorically stated that "yellow fever goes practically no farther than the traffic by sea, the disease for the most part finds its limits where that medium of communication ends" The evidence goes to prove that the windjammer of bygone days provided more favorable surroundings for the transit of infected mosquitoes than does the modern liner. It was repeatedly proved that *Aedes* could live for months and even breed in the humid, warm, ill-ventilated compartments of the old-time sailing vessels. In the case of the modern ocean liner, with the exception of rain water in lifeboats, there are few breeding grounds for mosquitoes on board, since there are no uncovered water tanks and the introduction of forced-draught ventilation tends to drive the insects out of the ship With regard to sailing vessels, the conditions appear to have been particularly bad on the Finnish and Swedish craft, and early writers such as Lallemant (1857) and others were loud in their condemnation of the filthy state of these boats Many bluntly attributed the disease to the insanitary living accommodation provided on the vessels, and Hirsch made the observation that yellow fever was chiefly to be found among the poorer classes who dwelt in dirty surroundings, amid the squalor and poverty-stricken quarters of human dockland habitations These early accounts make fascinating reading, for even although they do not blame insects as being possible vectors of infection, they are nevertheless interesting, since the experiences related are in accordance with our modern knowledge concerning the rôles of *Aedes aegypti* in the propagation of maritime and urban yellow fever For example, it has been noticed that the disease was sometimes limited to the crew of a ship, or that it was occasionally confined to one side of the vessel, or else to a single cabin. The presence of a high wind or cold weather experienced during the time of an epidemic were also said to modify its course. Indeed, it may be said that the early literature contains many shrewd observations which bear remarkable testimony to the accuracy of the facts as reported by pioneer workers

So often has yellow fever been transmitted by ship, that it has been popularly asserted that it has been sent backwards and forwards like a shuttlecock between Pernambuco and the Slave Coast of Africa. Under the circumstances, it is worth considering in detail the precise mechanism by which its spread is effected by sea We would again remind the reader, however, that although yellow fever is conveyed by ship, either through the transference of infected mosquitoes or human patients, a combination of factors is needed before the disease can establish itself permanently in any land As mentioned previously, 3 factors are essential for survival of the disease, namely, a human case of yellow fever, the insect vector *Aedes aegypti*, and last, but most important of all, the existence of susceptible individuals The theoretical possibilities of the spread of infection may be enumerated as follows (1) If an unrecognized case of the disease (during its incubation period) were to be landed at a port at which *A aegypti* were present together with a non-immune human population, a severe epidemic might ensue Should, however, *A aegypti* be absent, then it can be assumed that there is no likelihood of further human cases occurring in the district (2) The transportation of infected *Aedes* from one port to another forms the major problem of these two The presence of infected mosquitoes on a vessel may spread the condition in a variety of different ways Either the insects may infect susceptible sailors on board, or else those who come aboard the ship at its port of destination. If the temperature of the latter is favorable, the mosquitoes may leave the vessel and attempt to form a local colony

The Atlantic Islands.

The name of the island of St. Thomas, which lies about 200 miles off the coast of West Africa in the Cape Verde group of islands, appears to have figured prominently in the literature. Although today these islands are healthy places, free from yellow fever (see Colonial Office Report, 1920), at one time they seem to have been an endemic center of the disease, and there are numerous reports to the effect that St. Thomas was a notoriously unsalubrious spot. Sir Richard Hawkins (1622), [illegible] South Seas, alluded to the Cape Verde [illegible] are held to be scituate in one of the [illegible] therefore it is wisdome to shunne the sight of them, how much more to make abode in them. . . . In two times that I have been in them either cost us the one halfe of our people with Fevers and Fluxes of sundry kinds . . . and in one of them it cost me six monthes sicknesse with no small hazard of life." In 1585 Drake and Carleill captured the island, and spent 10 days on it, but after returning to their ships some days later many of their sailors developed a fever, as a result of which 300 of them died at sea. From Bigges's (1652) description of the signs and symptoms seen and complained of by Drake's sailors, Carter has identified the condition as being yellow fever. Other European expeditions to the island likewise suffered from the disease, which was prevalent during the time of the Brazilian wars that were fought between the Portuguese and the Dutch in 1624. Thus, both Menezes, who visited the island in 1624, and Mascarhenas, in 1638, suffered loss from its ravages.

Between the years 1581 and 1640, St Thomas was employed as a regular port of call for Portuguese and Spanish merchantmen trading between Europe and the New World, as well as for ships of other nationalities. The island was, moreover, the center of the slave trade and in regular communication with the African coast. St. Thomas was first colonized in 1485 and it is highly probable that, after yellow fever was imported into it from the mainland, it remained a permanent endemic focus for dissemination of the disease to the Western hemisphere. The forgoing facts pertaining to this island are not merely of historical interest but of great epidemiological significance, in so far as they clearly illustrate the requisite factors essential for the establishment of an infected area. Here the insect vector *Aedes aegypti* was present (either being found on the island before its colonization or else introduced into it by ships from the mainland), human cases of the disease were plentiful and, most essential of all, the constant influx of highly susceptible European sailors helped to swell the proportion of infected *Aedes* mosquitoes. A combination of favorable circumstances for the perpetuation and maintenance of infection thus appears to have existed on this island

The Canary islanders suffered repeatedly, and epidemics were reported from 1701 to 1888. From all accounts, it seems logical to conclude that, in early times, certain of the Atlantic islands which were used as ports of call by ships played a conspicuous part in the dissemination of yellow fever

Africa.

Lind (1792), an authority of his day on tropical diseases, described a condition not unlike yellow fever among sailors stationed on the sloop *Merlin* as it lay at anchor in the Gambia River in August, 1768. He stated that only men who had gone inland to fell wood in the forests contracted the disease, whereas those who had remained on board were unaffected In the following year, in 1769, the same fever was again observed by Lind to attack sailors attached to the *Weasel* and the *Hound* when lying in the same waters

Schotte (1782) studied the disease from 1775 to 1778 at St Louis de Senegal and presented a summary of his findings in the text of a report to the Royal Society, titled *Synochus Atrabiliosa* Yellow fever was next reported in Boullam and

ETIOLOGY

The progress made during recent years in the etiology, diagnosis, and prophylaxis of yellow fever may well be classed among the greatest achievements of modern medicine. During the first 2½ centuries following its appearance, man had been powerless to combat its ravages, as the mode of spread and origin were completely unknown. It was not, however, until 1881 that Carlos Finlay (1881, 1904, 1912) of Havana first suggested that infection was spread by a mosquito, and later, in 1900, that the classical researches of Reed and Carroll (1902) definitely established it as a virus disease which was transmitted by *Aedes aegypti*.

In these early times, before prophylactic vaccination was known, research work on yellow fever was attended with great personal risk to investigators, as a result of which many distinguished medical men lost their lives, one was Hideyo Noguchi, the celebrated Japanese bacteriologist, whose romantic life story has been narrated by Eckstein (1931).

Noguchi deserves special mention, as, in 1925, when working in South America, he isolated *leptospira* from cases of jaundice believed to have been cases of yellow fever by the local clinicians, but which in reality were Weil's disease Noguchi

he had been misled by cliniciar-
diagnosed cases in America. U
since before he could publish
victim to yellow fever and di
shared his unhappy lot were
Paul A. Lewis, and Theodor
posterity in the history of bacteriology. No reference to the subject of yellow fever would ever be complete without reference to the prominent part played by members of the International Health Division of the Rockefeller Institute, and in the following pages are included some of their many accomplishments

MODERN ANTIMOSQUITO MEASURES IN THE PREVENTION OF EPIDEMIC YELLOW FEVER

For effective mosquito control Public Health legislation is required, and much of the success in any district depends not only on the willingness of the inhabitants but also on the nature of the laws Thus, the rules should be drafted by ex-
ites of mosqui-
with sufficient
the town and,
if necessary, to force uncooperative owners of premises to carry out such structural alterations to their buildings, as may be deemed necessary to eliminate the breeding places of mosquitoes The value of antimosquito laws may be enhanced by the incorporation of clauses making the mouse protection test and viscerotome postmortem examination compulsory under circumstances where the cause of death is unknown One of the most comprehensive series of antimosquito regulations devised, and one well worth reading, is to be found in the Decree 21,434 of May 23, 1932, Approving the Regulations of the Services for the Prevention of Yellow Fever in Brazil. Without exception, in every country in which active antimosquito laws have been applied, there has been a corresponding decline in the incidence of epidemic yellow fever (Low, 1920)

on shore. This actually occurred in France at St. Nazaire in 1861, when the ship *Arequippa*, which called at the port, became infected in this way by mosquitoes from a neighboring vessel. On the other hand, if the shore temperature conditions are uninviting, the *Aedes* may remain in the vicinity of the ship and bite workmen occupied on the quay sides and docks lying within their range of flight Thus the outbreaks which occurred in Halifax in 1842 and 1861, and in Portland, Maine, in 1801, were ascribed to this cause (Carter, 1931). Similar localized epidemics occurred at Swansea in Great Britain in 1864 and 1865 (Buchanan, 1866), and at Southampton in 1866 and 1867 (Wiblin, 1866–7), when laborers and customs officials who had come into contact with infected ships contracted the disease.

During the years 1700 to 1878, according to Hirsch (1883), localized outbreaks and sporadic cases were reported from the following European localities.

Cadiz	1700, 1730–1, 1733, 1764, 1780, 1800, 1804, 1810	Andalusia	1819–21
Lisbon	1723	Granada	1819–21
Oporto	1851, 1856 (120 cases)	Tortosa	1821
Malaga	1741, 1803, 1804	Barcelona	1821, 1870
Madrid	1878	Brest	1802, 1839, 1856
Cartagena	1810	St Nazaire	1861
Gibraltar	1810, 1828	Swansea	1843, 1851, 1864, 1865
		Southampton	1852, 1866, 1867

There is another possibility, to which Carter (1931) has drawn attention, that illustrates how effectively infected mosquitoes can be locked up in ship compartments, and start disease only at the end of a long voyage. He cites several instances in which this happened. For example, in 1890 when the *Curlew* arrived at Ship Island Quarantine Station after a 58 days' voyage from Rio, although there had been no illnesses during the voyage, one of the seamen developed yellow fever 4 days after he had entered one of the ship's hatches. On the second occasion, when the British bark *Chippewa*, which had arrived from Rio after spending 68 days at sea without any history of illness on board, reached Ship Island, 2 cases of yellow fever developed on board

The foregoing experiences show how easily infected mosquitoes can be transported at sea They furthermore reveal that the apparent absence of illness on board a vessel by no means implies that it is free from disease, as the infected insects aboard the vessel may not have had access to the crew during the voyage, or else if they did so, the men may have been immune to infection

Before leaving this topic, we should like to refer to the Madrid outbreak of 1878, which presented certain unusual features and illustrated how contaminated baggage could convey the disease by railroad transport far into a country. The associated circumstances were as follows In 1878 a regiment of soldiers, who had previously been in excellent health, returned to Santander from Cuba About one-half of the men proceeded directly from Santander to Madrid by railway train, conveying with them their unopened kit, which was unpacked and cleaned at Madrid for the first time since they had left Cuba Soon afterwards cases of yellow fever developed among members of the civil population, whereas the troops continued to remain unaffected Guichet (see Hirsch, 1883), who described this epidemic, stated that "Yellow fever at Madrid was due to the importation of morbid germs among the clothes and baggage of men in good health returning from Cuba" Although the existence of mosquitoes was never alluded to in this account, the essential facts were so graphically narrated, that the cause of the epidemic could have been due to none other than to infected *Aedes* present in the troops' baggage

The fact that only the civilians suffered is also easily explained, as the troops were probably immune to infection by previous exposure to attack when serving abroad, whereas the inhabitants of Madrid were unprotected, and therefore highly susceptible to infection.

there has not been a single case on record where infected mosquitoes or patients have been transported by air.

Admittedly the airplane may be a potential source of danger, but simultaneously the boundaries of human yellow fever have been pushed backwards and the advent of DDT ... ledge of the ... minimized ... fully aware ... l action of DDT on the upholstery of airplanes has been studied by Madden, Lindquist, and Knipling (1946) who concluded that DDT was a useful adjuvant to normal spraying which it should reinforce but not replace.

INDIA AND THE DANGER OF YELLOW FEVER

The late British Government of India was conscious of the danger of the entry of infected *Aedes* or human cases of yellow fever through the airport of Karachi

Sprawson (1936) emphasized the point that in India there existed a large population of nonimmunes to yellow fever, coupled with an abundance of the insect vector *A. aegypti* in the country. For example, in Calcutta alone the index of *A. aegypti* was estimated to be 12 6 per cent, by the researches of Chowdhury and Ganguli (1937) who collected 2,624 mosquitoes from 20,825 dwelling houses in the city. An Indian strain of *Aedes aegypti* has been shown by Hindle (1929) to be capable of transmitting yellow fever. There is, therefore, no justification for any supposition that the absence of yellow fever in India is due to a biologically different species of *Aedes aegypti* in that country which is incapable of functioning as a vector Another potential carrier of infection is *Stegomyia vittata* (*sugens*) which is widely distributed throughout Ethiopia, Aden, the surrounding hinterland, and India. If yellow fever were introduced into India, the consequences would be too terrible to imagine, for, in addition to the factors already mentioned, it must be remembered that the Indian *M rhesus* monkey is highly susceptible to the disease and would constitute another source of danger To prevent the introduction of the disease, Sprawson proposed at the Pan-African conference that the following ...

tained by appropriate methods, (3) that the personnel of all aircraft be actively immunized against yellow fever (see p 503).

As yet, there is no record in the literature of yellow fever being transported by air to any new locality. This has doubtless been due to the efficiency with which the authorities concerned have tackled the problem, and if recommendations such as those suggested above are rigorously enforced, there is no reason to fear that infected mosquitoes will be conveyed by air. The transport of unrecognized human cases of yellow fever (for example, during the incubation period) is, however, a more dangerous and less controllable factor To take a hypothetical case, a susceptible individual in good health may be bitten by an infected *Aedes* on the morning of his departure by airplane from West Africa. A few hours later the person may arrive at Karachi, pass through the airport, and later develop the disease elsewhere in the country. Admittedly this is an extreme example, but it is nevertheless a contingency worth entertaining, if it is remembered that to-day it is possible to travel from England to Australia in less than a week Theoretically, a period of 7 days' quarantine for all air passengers would be the ideal means of controlling infection such as that alluded to above, but unfortunately the introduction of quarantine would defeat the advantages gained from speedy air travel!

The transport of unrecognized human cases of endemic yellow fever is another problem which has some bearing on this subject Hoffmann (1937) states that

Dichloro-diphenyl-trichlorethane (DDT) $(ClC_6H_4)_2$ $CHCCl_3$

The insecticidal efficacy of DDT is too well known to warrant further description. Webster (1946) recommends that mosquito breeding marshes should be sprayed with a solution of DDT 20 per cent, triton 20 per cent. and xylene 60 per cent., diluted with water to form a 5 per cent. final concentration of DDT in aqueous emulsion. One to 2 quarts per acre suffices. A dilution of one part per million prevents breeding of mosquito larvae for several weeks and a concentration of 25 mgms per sq. ft., 100 days after application kills adult mosquitoes in 4 hours. At a concentration of 50 mgms per sq ft. after a lapse of 308 days mosquitoes are killed in 24 hours.

Although DDT displays great powers in persistence, its immediate lethal effect (in low concentration) is small and to overcome this defect DDT is combined with pyrethrines such as in the very successful American aerosol bomb. (See review by Baillie, 1946.)

According to Elmendorf, Marucci, Griffin, Myer and Ryan (1946), tests on 18 mesh per inch wire screens painted with 200 mgs. DDT per square foot, showed that 30 minutes after 4 contact exposures, some 61.1 per cent. *Aedes aegypti* failed to bite. Domestic species of mosquitoes in the hunt for food would presumably rest on the walls of houses prior to biting the inhabitants. There is still opportunity for further research on the choice of solvents, crystallization effects, and the action of weathering.

DDT promises to be an important weapon in the control of *Aedes aegypti* wherever it may occur. For instance, at Georgetown, British Guiana, in spite of heavy rainfall in April–June the application of DDT successfully reduced the *Aedes aegypti* house index (for adults, pupae, and larvae) from 2.19 per cent. at the end of 1945 to 0.32 per cent. at the end of June, 1946. A single spraying of the walls of houses with 5 per cent. DDT in kerosene remained insecticidal for 10 months (de Caires, 1946).

STEAMSHIPS AND THE SPREAD OF YELLOW FEVER

The modern ocean liner is less likely to convey yellow fever from one port to another than the old-time sailing vessel because *Aedes aegypti* is a comparatively delicate insect which avoids air currents and temperatures below 65° F. (see Low, 1920). Furthermore, as modern vessels are equipped with steam water condensers there are no accessible water barrels or similar storage receptacles in which mosquitoes can breed. It seems improbable that steamers can convey infection to any extent, and at the Paris International Sanitary Conference (1911–12) it was emphatically stated that "there was not a single instance on record where yellow fever had been conveyed overseas after a long voyage, on a steam vessel to another country, unless an actual human case or cases of the disease had previously occurred during the voyage." Thus the chances of yellow fever being transmitted from South or Central America, across the Pacific over a distance of 11,000 miles, to Calcutta in India, are remote.

AIRCRAFT AND SPREAD OF YELLOW FEVER

According to Findlay (1946), the present organizations for the prevention of yellow fever and other diseases by aircraft are unsatisfactory. Mosquitoes have been proved to survive journeys of 9,000 miles at heights of 10,000 to 12,000 feet. Findlay believes that the sanitary conditions of airfields, aircraft, air passengers, crews, and ground staffs may be inadequately supervised and that yellow fever immunization certificates may be an ineffective check through evasion, forgery, or faulty preparation, dilution, and administration of vaccine. Notwithstanding the many opportunities for wilful evasion or unintentional neglect of official instructions, it is perhaps true to state that throughout the war years and postwar period,

permitting of its development, inoculation of such personnel is recommended. (6) That all persons inoculated in compliance with the provisions of paragraphs (4) and (5) of this Article shall be furnished with and carry an Inoculation Certificate signed by the officer carrying out the inoculation This certificate shall conform to the International Form of Certificate of Inoculation against yellow fever annexed hereto (7) That persons in possession of a valid antiyellow fever inoculation certificate shall not for the purpose of the control of yellow fever be subjected to quarantine restrictions. (8) That in place of a valid antiyellow fever inoculation certificate, a certificate that the bearer has recovered from an attack of yellow fever and that his blood contains immune bodies against yellow fever, as proved by

, for yellow

concerned,

ellow fever

risk of contracting yellow fever during the period of his stay in an endemic yellow fever area. (10) That UNRRA shall lay down standards with which yellow fever vaccine shall conform. (11) That they will make arrangements to test at frequent intervals the activity of the yellow fever immunizing vaccine in use in order to insure that its immunizing properties are satisfactory, and for this purpose agree that UNRRA in consu

Western Hemisphere,

time to time institutes

Article 37 require

UNRRA

Article 38 deals with provisions concerning regions in which yellow fever is discovered or exists in endemic form. Salient features are that aerodromes be situated in areas where water supplies and human dwelling quarters can be made

are that all aircraft should be disinsected, travelers not in possession of the official yellow fever inoculation certificate are to be isolated in screened quarters for 6 days

Article 49 deals with territories or regions where conditions do not permit of yellow fever developing Aircraft may land on any authorized aerodrome, the plane be disinsected and the occupants inspected medically.

Ships

The following are 2 of the regulations laid down by the International Sanitary Convention (1926) amended 1944

"Article 35 A ship shall be regarded as *infected* if there is a case of yellow fever on board, or if there was one at the time of departure or during the voyage. A ship shall be regarded as *suspected* if it had no case of yellow fever but arrives after a voyage of less than six days from an infected port or from an uninfected port in close relation with endemic centers of yellow fever, or if when it arrives having been more than six days out, there is reason to believe that it may carry winged *Stegomyia* (*Aedes aegypti*) from the said port. A ship shall be regarded as *healthy*, notwithstanding its having come from a yellow fever infected port, if, having had no case of yellow fever on board and arriving after a voyage of more than six days, there is no reason to believe that it carries winged *Stegomyia*, or when it proves to the satisfaction of the sanitary authority of the port of arrival (*a*) that during its stay in the port of departure it kept at a distance of at least 400 meters from the inhabited land and at such a distance from the pontoons as to make the access of *Stegomyia* improbable, (*b*) or that at the time of departure it was subjected to effective fumigation in order to destroy mosquitoes

endemic yellow fever in man is associated with slight, noncharacteristic manifestations of illness which may sometimes be unrecognizable clinically. Such cases in the past, he says, have escaped detection, and are to-day being found as the result of routine viscerotome examinations of all cadavers in different countries If this is so, then it should be a comparatively easy matter for an individual suffering mild endemic yellow fever to be transported by air to a locality where the disease does not occur. Subsequently, if the latter country contains the insect vector as well as nonimmune inhabitants, the disease may break out with epidemic violence. Such a possibility has already been appreciated, and appropriate legislation has been enforced by the Government of India to deal with such an eventuality In a publication dealing with this subject, Chowdhury and Ganguli (1937) have discussed the problems involved in the control of *Aedes aegypti* in Calcutta, with reference to dengue fever and the possible importation of yellow fever into India by air, and give particulars of the notification of the Government of India regarding yellow fever, which has been framed in the following terms:

"In exercise of the powers conferred by section 2 of the Epidemic Diseases Act 1897 (III of 1897), the Governor-General-in-Council, being satisfied that India is being threatened with an outbreak of Yellow Fever, is pleased to direct (1) that no person shall arrive in India by an aircraft within nine days of his being in an area in which Yellow Fever exists, or in which though the disease has not been clinically demonstrated there is presumptive evidence, as a result of mouse-protection tests, that the disease has occurred in that area, unless he has been protected by satisfactory inoculation, or previous attack of the disease, and (2) that no aircraft which started from or alighted in any such area shall enter India."

LEGISLATION FOR PREVENTION OF SPREAD OF YELLOW FEVER

Aircraft

Official regulations designed to prohibit the spread of infection are to be found in the articles of the International Sanitary Convention for Aerial Navigation, 1933, amended, 1944 These are so extensively circularized by the Epidemiological Information Bulletin of United Nations Relief and Rehabilitation Administration (UNRRA) that it is only necessary to quote the general provisions of regulations applicable to man in Article 36.

"(1) That persons suffering, or suspected to be suffering, from yellow fever shall not be allowed to embark on aircraft on international flight. (2) That they will take all possible measures to establish the existence or nonexistence of yellow fever within their territories For this purpose, in territories where endemicity of yellow fever is suspected, in cases where the person dies within 10 days from the onset of any undiagnosed febrile illness, it is important that a specimen of liver tissue be taken, if necessary by viscerotome, for histopathological examination In endemic areas a sample of blood for a yellow fever immunity test should, in addition, wherever possible, be taken from all persons suffering from an undiagnosed fever, and if the cause of the fever remains doubtful and the patient recovers a second sample should be collected at the end of the third week from the onset of illness (3) For the purpose of quarantine control, UNRRA in consultation with the governments concerned and, as regards the Western Hemisphere, with the Pan-American Sanitary Bureau, shall define the boundaries of endemic yellow fever areas (4) That they shall use their best endeavors to secure that all persons who are likely to land in an endemic yellow fever area shall be inoculated against yellow fever 10 days before arrival in the area and that, so long as such persons remain in the area, they shall be reinoculated every 4 years (5) (*a*) That inoculation against yellow fever shall be required for all regular staff employees and crews using authorized aerodromes situated in endemic yellow fever areas (*b*) That in areas in which yellow fever does not exist, but in which there may be conditions

CHAPTER XXXIX

CLINICAL MANIFESTATIONS OF YELLOW FEVER

CLINICALLY, yellow fever manifests itself as a single entity. For epidemiological purposes, outbreaks have been classified into different varieties according to the geographical surroundings in which the disease has been acquired. Hence the term "epidemic yellow fever" has been used to describe large outbreaks, affecting communities, caused by the insect vector *Aedes aegypti* (*Stegomyia fasciata*). The terms "urban" and "rural" have been applied to cases contracted in town and country respectively, whereas in the past the name "maritime yellow fever" has been reserved for outbreaks associated with ships and seaport areas Within comparatively recent times 3 new epidemiological descriptive terms have been added to the literature, namely the so-called "endemic," "sylvatic" and "jungle yellow fever." The former term has been designated for those cases occurring in the interior of South America, which once was thought to be free from the disease but is now known to be infected.

GENERAL CHARACTERISTICS

In tropical climates, cases are liable to occur throughout the year but in subtropical zones they are limited to the summer months Individuals visiting the forests and nonimmune new arrivals in endemic zones are vulnerable to attack.

Age and Sex Incidence

During the 1921 epidemic in Peru, Hanson (1929)[1] reported that the highest death rate occurred among young children from 1 to 3 years of age, and the mortality among this group was used to determine the endemicity of the disease at various centers. The death rate was lowest in those aged 5 to 15 years, and males appeared to be slightly more affected than females. In individuals over 50 years of age the prognosis was grave, and death usually occurred about the 6th day after the onset of symptoms.

Clinical Signs and Symptoms

The onset may be either sudden or insidious, being ushered in with a rigor in an individual who has previously been in good health, or else it may commence with an initial period of malaise. The subsequent course of the disease is usually divided into 3 clinical stages that exhibit different manifestations. These are (1) the initial fever, (2) the period of calm, and (3) the stage of reaction, if the attack is a severe one.

present The disease is highly infective in its early stages

Stage 2 is marked by the commencement of jaundice, it lasts from 1 to 3 days, the pulse and temperature may return to normal and complete recovery may result. In unfavorable cases vomiting of black colored blood and suppression of urine may supervene Such cases pass into stage 3, namely that of reaction. This may last about 7 days and the patient is usually critically ill. The temperature may rise

[1] References are appended at the conclusion of Ch. XLIII

"Article 36. Ships *infected* with yellow fever shall undergo the following measures: (1) Medical inspection. (2) The patients shall be landed, and those of them who are in the first five days of the illness shall be isolated so as to prevent infection of mosquitoes. (3) The other persons who land shall be subjected to observation or surveillance not exceeding six days reckoned from the time of landing (4) The ship will be moored at least 400 meters from the inhabited land and at such a distance from the pontoons as will render the access of *Stegomyia* improbable. (5) Mosquitoes at all stages of evolution shall be destroyed on board as far as possible before discharge of cargo. If unloading takes place before the destruction of mosquitoes, the personnel in charge of that work will be subjected to observation or to surveillance for not more than six days from the time when they ceased unloading"

CLINICAL PATHOLOGY

Albuminuria occurs early in the course of the disease and, when present, is of diagnostic value in doubtful cases, later on, the urine becomes reduced in quantity and may contain up to 2 gm of albumin per liter Additional data concerning the abnormal constituents of the urine in yellow fever are to be found in the work of Pichat (1929). Blood is present in the characteristic black vomit, as well as in the stools. A polymorphonuclear leukocytosis occurs in the initial stages of the illness and is later followed by leukopenia. Granular casts were present in the urine

Blood The percentage of polymorph leukocytes varied considerably, though in many cases their numbers were reduced. Lymphocytes were relatively increased. Hypoglycemia is often present and a rise in *blood guanidine* has been reported by Soper (1943)

DIFFERENTIAL DIAGNOSIS

In acute cases, the diagnosis is easy, and must be differentiated from other tropical diseases such as smallpox, measles (often fatal in colored races), influenza, dysentery, plague, pneumonia, Weil's disease, relapsing fever, typhus, scurvy and blackwater fever. In mild cases of yellow fever the disease is sometimes hard to identify, and the differential diagnosis from dengue fever has to rest on the results of leukocyte counts. Infective hepatitis can usually be excluded by the absence of marked constitutional signs and symptoms Carbon tetrachloride poisoning following antihelminthic treatment must be eliminated

MORBID ANATOMY OF YELLOW FEVER

The principal changes found at postmortem in a fatal case occurring in a nonimmune European (or other) subject are as follows

Icterus, hemorrhages into various organs, hemorrhages into the mucous membrane of the stomach; necrosis of the liver, degeneration of kidney tissue and discoloration of the urine Hindle (1930 *a*) states that the jaundice found at autopsy is lemon yellow in color, is conspicuous on the skin, conjunctivae, cartilages, the region of the larynx, over the great vessels of the heart, and, moreover, that the fat and loose tissues of the body are likewise discolored

[illegible]

necrosis The kidney [illegible]
albumin, bile, hyaline [illegible]
malpighian bodies are [illegible]
ent hemorrhages.

MORBID HISTOLOGY

Liver lesions are characteristic and diagnostic of the disease The changes have been described by Rocha Lima (1912), Hudson (1928), Torres (1928), Klotz and Belt (1930), and Hoffmann (1928, 1937) According to Bablet and Bloch (1937), tissues should be placed in Duboscq–Brasil's liquid for 24 hours, then embedded in paraffin, sectioned 5 μ thick, and stained by the following method. Mayer's hemalum 10 minutes, wash in water, differentiate in 90 per cent. alcohol containing 5 drops of HCl per 100 c c for a few seconds, wash in tap water for 5 minutes, stain in 1 per cent bluish eosin for 3 minutes, wash repeatedly in tap water. Stain and differentiate in alcoholic solution of safranine for 3 minutes, dehydrate in absolute alcohol rapidly, thereby removing the excess of safranine, treat with toluol, and mount in neutral Canada balsam

again, the pulse rate fall, and black vomiting develop. Prostration, anuria, melena, jaundice, and epigastric pains increase in severity, and death may end the scene Sometimes, before the end, indications of cerebral involvement such as delirium, convulsive spasms, and coma may become evident.

In cases which recover, the individual usually commences to improve about the 8th day after the beginning of illness, and thereafter convalescence may proceed in an uninterrupted fashion Relapses are frequent and dangerous when they occur Recovery from one attack confers lasting immunity against reinfection

There is no specific treatment for yellow fever.

YELLOW FEVER IN THE WEST AFRICAN NATIVE

The disease probably assumes a milder form in the African Negro Beeuwkes (1937) gives a good account of the clinical manifestations of the disease as he studied it during the course of 4 extensive epidemics affecting the Gold Coast and Nigeria Beeuwkes points out that in the past, the description of the disease was almost exclusively confined to its occurrence among Europeans, as cases among Africans were rarely observed except when an epidemic took place among the whites The number of cases reported among the black population has been relatively small, and the symptoms of the disease are often of so mild a character that extensive epidemics affecting the native population have frequently escaped recognition.

Clinical Findings

Males appeared to be more commonly attacked than females, and the death rate varied from 10 to 32 per cent. among individuals between 30 and 51 years of age

The duration of illness was usually from 8 to 9 days, and in fatal cases death occurred on the 7th day, being later in comparison with the time of death in the European subject.

Temperature lasted about 3½ days, it tended to be high at the commencement of the illness and thereafter gradually declined In mild cases the temperature reached about 104° F with a pulse of 120 per min In such cases Faget's pulse was absent, but a relatively slow pulse was observed in about 40 per cent. of instances

According to Maegraith (1946), Faget's description mentions 2 signs (1) an initial diagnostic sign, slowing pulse rate with rising temperature, and (2) a final evil prognostic sign, quickening pulse rate with fall in temperature

Headache and bodily pains were invariably present *Anorexia* was a prominent symptom *Vomiting* was marked only in the severe cases, but was of a transient character in the milder infections *Epistaxis* was rarely seen, and bleeding gums were less common than in the European *The degree of prostration* evident in different cases varied considerably

Congested eyes were generally observed at the onset of the illness and persisted for 4 to 5 days

Icterus manifested itself by the presence of a slight golden tinting of the sclerae, and, when pronounced, it extended to the mucous membranes of the conjunctivae, mouth, and lighter cutaneous areas

Liver Tenderness was present, but the extent of enlargement of the organ could not be accurately elicited

The spleen was observed to be hard and enlarged, but whether or not this was due to the effects of malaria could not be determined

Tongue Beeuwkes stated that a tongue typical of yellow fever is "small, more or less pointed, with a white or brown coat over the division and definite red edges and tip . . ."

Albuminuria was present in every case and varied greatly in intensity.

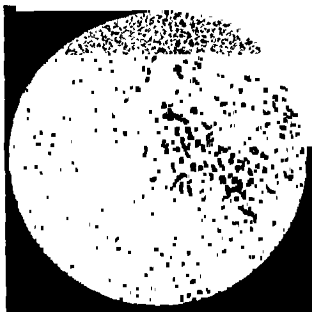


Fig 32 Photomicrograph of an histological section prepared from liver tissue removed from a fatal case of yellow fever. Note the extensive fatty degenerative change and the few recognizable liver cells Stained hematoxylin and eosin X90

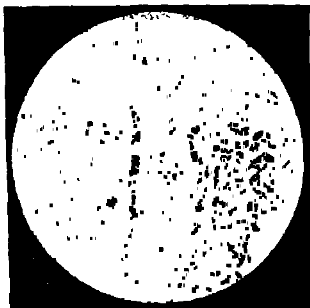


Fig 33 Histological section of kidney from a fatal case of yellow fever Note the lime cast in the center of the field Stained hematoxylin and eosin X90

An affected tissue section shows extensive coagulation necrosis, with fatty degenerative changes commencing in the mid-zonal area and extending to the portal and central veins (Rocha Lima, 1912). Often there are left intact a few layers of parenchymatous cells around the central vein in the peripheral parts of the lobule. The liver cells are extensively vacuolated, separated by spaces, and are markedly acidophilic in staining affinity. Here and there in the affected cells there appear large, rounded, granular, necrotic masses which have been named Councilman lesions, and a description of these is to be found in the work of Babler (1936, 1937) The nuclei of individual liver cells are damaged, being either disintegrated or showing such injury as loss of staining property margination of the chromatin, or the presence of acidophilic-staining material at their center. The latter have been called *the intranuclear inclusion bodies of Torres* (1928), and are most conspicuous in the early stages of yellow fever prior to the onset of advanced liver changes (see Cowdry and Kitchen, 1930).

Nicolau, Kopciowska, and Mathis (1934 *a*) studied the morphology of these inclusions, and found that they were oxyphilic bodies that lacked any internal structure and were sometimes surrounded by a halo in the karyoplasm In size, they varied from 1 to 4 μ in diameter, and several of them often appeared in a single cell These workers believed that the inclusion body represents the reaction of the nucleus against the virus which has entered the cell.

In addition to the above findings, minute hemorrhages are distributed throughout the liver parenchyma, the sinuses contain disintegrated leukocytes, and Kupffer cells may be seen lining their boundaries. The damage done to the Kupffer cells in yellow fever has been studied by Klotz and Simpson (1927), who reported that hyperplasia of these stellate cells was to be found in the late stages of the less severe cases, and their presence appeared to be associated with the existence of clinical jaundice. These cells also revealed other changes such as granular degeneration, pyknotic nuclei, and the presence of bile or iron pigment within their cytoplasm. In a later publication Klotz and Belt (1930) examined histological sections of liver derived from 93 fatal cases of human yellow fever, and declared that the vascular system, biliary channels, and stroma were unaffected in the disease.

In general it may be stated that the liver changes in yellow fever closely resemble those of acute yellow atrophy, and considerable experience is required on the part of the pathologist to arrive at a diagnosis of yellow fever from the results of microscopical examination alone (see Fig. 32)

The kidney Acidophilic necrosis of the tubular epithelium with fatty degeneration is present The glomeruli are congested and the renal tubules may contain hyaline, granular, or calcareous casts. Hoffmann (1937 *a*) attaches special significance to the existence of renal lime casts in yellow fever as a point of diagnostic value in suspected cases of the disease (see Fig 33). The general appearance presented by the organ as a whole is that of an acute toxemic reaction.

The regeneration of liver and kidney tissue following an attack of yellow fever has been studied in rhesus monkeys and is referred to in the appropriate section on p 499, describing the lesions produced in these animals

The brain Nicolau, Mathis, and Baffet (1937) examined histological sections of brain, prepared from a fatal case of yellow fever, which were stained by Mann's method, and recorded the following findings The meninges were normal, but there was proliferation of glial cells in both gray and white matter around the blood vessels Perivascular mononuclear infiltration was present around blood vessels and sometimes also surrounded isolated nerve cells Both the nerve cells and glial cells of the cerebral cortex contained eosinophil intranuclear inclusion bodies, and some of the latter were surrounded by a halo of nonstaining material. Lesions similar to these were also detected in the brains of monkeys, guinea-pigs, and mice after experimental infection with yellow fever virus (Nicolau, Mathis, and Baffet, 1936). The histopathology of the brain and spinal cord of 14 fatal cases was con-

reactions and serum protection tests have also proved that no antigenic differences exist between the yellow fever virus of the western hemisphere and that of Africa (Theiler and Sellards, 1928, Hudson, Philip, and Davis, 1929; and Hudson, Bauer, and Philip, 1929).

The Value of Routine Liver Examination and Use of the Viscerotome

The *viscerotome is a metal instrument (see Fig 34) resembling a trocar, about* 1 cm. square, possessing a sharp, hollow, pointed extremity fitted with a mechanically operated guillotine blade. Thus, by plunging the sharp end into the right epigastric region of a cadaver it is possible to extract a piece of liver tissue without the *inconvenience of having to perform* a postmortem examination. Specially constructed viscerotomes are also available for obtaining blocks of kidney tissue or other organs Recently, in South America, a regular viscerotomy service has been inaugurated, and legislation has been introduced whereby officials of the yellow fever service have been authorized to remove specimens of liver from all persons dying after any illness of less than 10 days' duration (see Soper, Rickard, and Crawford, 1934).

Soper states that up to 1936 more than 65,000 viscerotomies have been per-

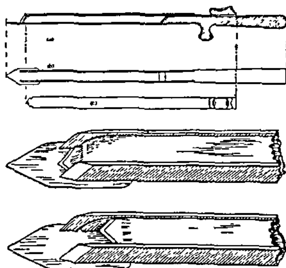


FIG. 34 Diagram illustrating the construction of the viscerotome

(Reproduced from Soper, Rickard, and Crawford, 1934, Amer J Hyg, 19, 549)

formed in about 1,500 places in Brazil, Bolivia, Paraguay, Ecuador, and Colombia.

The *outcome of the routine* examination of liver tissue from all fatal pyrexias information, thus justify- the existence of yellow uzed, and cases are discovered among natives, foreigners, adults, and in children resident in urban and jungle areas, situated both within and without known endemic localities (see Rickard, 1937).

ducted by Stevenson (1939). The principal lesions were perivascular hemorrhage affecting the subthalamic and periventricular region at the level of the mamillary bodies The temporal pole and cerebellum were involved to a lesser extent and similar changes were seen in the hippocampus and lumbar region of the spinal cord. Perivascular edema and sclerosis of ganglion cells of the superior frontal gyrus also occurred.

The pituitary body is engorged with blood, the tissue is markedly degenerate, and petechial hemorrhages are scattered throughout the white substance (Marchoux and Simond, 1906 *b*).

Heart. This organ was examined in 29 fatal cases of yellow fever occurring in West Africa by Cannell (1928), and cloudy swelling and fatty degeneration were the most constantly found features The distribution of the latter was patchy in character and most marked in the neighborhood of nuclei of the fibers Primary inflammatory changes were usually absent in yellow fever, but secondary response of white cells to intense degenerative changes was observed in 2 human cases The lesions occurring in the heart, when considered as a whole, are not sufficient to warrant a diagnosis of yellow fever being made on their presence alone From the results of his investigations into the state of the heart in yellow fever, Cannell is unable to find any reason to account for the existence of a slow pulse rate which is sometimes manifest in this disease. He believes that bradycardia is not due to concurrent jaundice and that examination of the bundle of His may conceivably shed some light on the problem (see also Marchoux and Simond, 1906 *c*)

Spleen Klotz and Belt (1930 *a*) noted the absence of hyperplasia in the fixed tissues of the pulp and observed the presence of certain changes in the malpighian corpuscles of the organ. In the latter, there were present mononuclear cells accompanied by reduction in the number of lymphocytes of the spleen, hyperplasia of the fixed tissues of the follicle, and waxy degeneration of the organ. A moderate degree of eosinophilia was detected in cases where the pathological lesions were advanced.

The pancreas, body *of the testicle, spermatic cord,* and *suprarenal glands* show
[illegible] *lacrimal, salivary,* and *thyroid glands* re-
[illegible] are less likely to occur in these organs
[illegible]

LABORATORY DIAGNOSIS

Sterile blood should be obtained within the first 3 days of fever, packed in ice, transmitted to the laboratory and inoculated subcutaneously or intraperitoneally in rhesus monkeys. In positive cases the latter develop fever after 3 days and may die thereafter exhibiting typical liver lesions On the first day of the patient's illness sterile serum should also be removed, the mouse protection test performed and the operation repeated 5 days later to ascertain if rise in antibody titer has occurred

The liver lesions found in a fatal case of yellow fever are diagnostic of the condition.

Tissue should be removed at autopsy, placed in 10 per cent formalin in physiological saline, embedded in paraffin, sectioned and stained by the methods described. The existence of the microscopic lesions mentioned previously is conclusive evidence of infection According to Bablet and Bloch (1937), the most diagnostic features are the presence of Councilman lesions, hyaline necrosis of liver cells, and total disappearance of nuclear chromatin. The finding of intranuclear inclusion bodies clinches the diagnosis of yellow fever, but it is not always possible to demonstrate these structures

The identity of American and African yellow fever The pathological lesions caused by the virus isolated in either country are similar in their general characteristics, but Klotz and Belt (1930 *b*) have pointed out that individual strains are sometimes liable to vary For convenience, it may here be noted that cross immunological

CHAPTER XL

YELLOW FEVER IN SOUTH AMERICA

Previous Epidemics[1]

BELOW is a record of outbreaks during the twentieth century in Central and South America, with dates of introduction of antimosquito legislation in different areas, and approximate numbers of cases of yellow fever prevalent at that time.

1893–1900 Havana, Vera Cruz, Isthmus of Panama, Rio de Janeiro
1900 Antimosquito measures were inaugurated at Havana (310 cases, population 300,000)
1902–3 Vera Cruz, Isthmus of Panama, Rio de Janeiro, Guayaquil
1904 Antimosquito measures were inaugurated at Vera Cruz (375 cases; population, 45,000)
1904–8 Havana, Vera Cruz, Isthmus of Panama, Rio de Janeiro, Para, Manaos, Guayaquil
1905 Antimosquito measures were inaugurated at Isthmus of Panama and at Rio de Janeiro (289 cases, population 1,000,000)
1907–9 Martinique
1908–13 Para, Manaos, Guayaquil
1910 Antimosquito measures were inaugurated at Para (358 cases, population 200,000)
1913 Antimosquito measures were inaugurated at Manaos (96 cases, population 60,000)
1913–17 Guayaquil (over 505 cases, population 54,000)
1915–16 Merida (Yucatan) Mexico, a few cases
1917 Merida and Peto (Yucatan) Mexico, a few cases only
1918 Antimosquito measures inaugurated at Guayaquil
1920–4 Mexico, Central America, Colombia, Peru, Brazil
1925–7 Brazil
1928 Rio de Janeiro, North Brazil
1929 South Brazil, São Paulo, South Para, at the mouth of the Amazon in the north The disease extended for over an area of 1,000 miles from Buenos Aires to Manaos. (A good example of so-called "maritime" yellow fever)
1929 Yellow fever reappeared in the interior of Colombia and Venezuela after an absence of 6 years
1929–30 Cases were recognized at only one point well away from the coast of Brazil in a well-isolated area
1930 The year marked the introduction of viscerotomy (Soper, Rickard, and Crawford, 1934), and the development of the mouse protection test (Sawyer and Lloyd, 1931)
1933 There were 3 confirmations of the possibility of yellow fever without *Aedes aegypti*, from widely separated areas at San Ramon (Bolivia), Lavro Sadre in Brazil, and at Caparrapi in the Magdalena Valley of Colombia.
1934 Yellow fever transmitted by *A aegypti* was relatively unimportant. Jungle yellow fever, on the other hand, was reported in Magdalena and Orinoco river valleys in Colombia (Soper, 1936), and at 3 points in Brazil at Para, Bahia, and Matto Grosso (Soper, 1936)
1935 Yellow fever transmitted by *A aegypti* was only proved to exist at one point in South America (in Minas Geraes, Brazil) (Soper, 1936)

[1] References are appended at the conclusion of Ch XLIII, p 511 et seq

Positive results indicate the exact spot at which infection exists, and so provide a starting point for the inquiries of the health authorities. Negative reports, on the other hand, are equally informative, as a long succession of negative reports from any one area indicates the absence of the disease. The value of viscerotomy in the diagnosis of other pathological conditions has been reported by Gast-Galvis (1945)

decline, Soper suggested that this was probably attributable to inefficiency of the insect vector rather than to a failure of human hosts. Subsequently Soper (1936) and others actively investigated the subject, and defined the jungle disease as "yel-



FIG 35 South American yellow fever area, delineated by Expert Commission on Quarantine. Since the publication of this map, minor changes have been made, particularly as regards the areas delineated in Brazil (*Epidem Inform Bull*, 1946, *2*, 580, 689, 772).

(Reproduced from *Epidem Inform Bull*, 1945, *1*, 687, by permission of the Executive Secretary, Interim Commission, World Health Organization.)

low fever occurring in rural jungle or fluvial zones in the absence of *Aedes aegypti*."

There are many points of distinction between epidemic or rural yellow fever and jungle yellow fever apart from the fact that the former is transmitted by *Aedes*, whereas the latter is not. Thus, yellow fever transmitted by *Aedes* is usually

1936 In this year James (1936) reported the occurrence of 16 cases in Bolivia, and 179 in Brazil

1944 Jungle yellow fever occurred in the state of Barinas Bolivar and Tachira, Western Venezuela (Diaz, 1945). So successful have been the anti-mosquito measures that in recent decades the boundaries of yellow fever have considerably regressed and in the year 1934 urban *Aedes aegypti* transmitted yellow fever was only present at a single center in Brazil Thus, the urban *Aedes* transmitted disease, one trusts, will not recur, and the problem of jungle yellow fever next awaits conquest.

GEOGRAPHICAL DISTRIBUTION

The potential distribution of yellow fever liable to arise from jungle sources based on clinical, pathological, and serological information is shown in Fig 35. (See de Paula Souza, 1945.)

"For the purpose of quarantine control, the areas in the South American continent, which for the time being should be considered as endemic yellow fever areas, are bounded by a line beginning on the Pacific coast of Colombia at the 5° N. parallel of latitude and extending east along that parallel of latitude to the eastern slopes of the Central Cordillera to an elevation of 2000 meters, thence southward along the eastern slopes of the Central Cordillera and the Andes Mountains, at the same elevation to the boundaries of Argentina and Bolivia, thence eastward along the southern boundary of Bolivia with Argentina and Paraguay to the 20° S parallel of latitude, thence east along that parallel in Brazil to the eastern border of the State of Mato Grosso, thence southward along the eastern boundary of the State of Mato Grosso to the 21° S parallel of latitude, thence east along that parallel to the 45° meridian of longitude, thence north along that meridian to the 15° S parallel of latitude, thence west along that parallel to the western boundary of the State of Goiaz, thence northward along that boundary of the State of Maranhao to the Atlantic coast, thence along the Atlantic and Caribbean coasts of South America to the eastern boundary of the Canal Zone, thence southward across the Isthmus of Panama and along the Pacific coast of Panama and Colombia to the 5° N parallel of latitude, excluding, however, the ports of Belem in Brazil, Cayenne in French Guiana, Paramaribo in Surinam, Georgetown in British Guiana, and the Caribbean ports of Colombia and Venezuela, and the cities of Caracas in Venezuela, Bogota in Colombia, and Corumba in Brazil

"In addition, the Ilheos and Itabuna Districts in the State of Bahia in Brazil bounded on the north by the River Contas, on the west by the 40° meridian of longitude, on the south by the River Pardo and on the east by the Atlantic Ocean, are for the time being, regarded as endemic yellow fever areas" (de Paula Souza, 1945)

ETIOLOGY OF JUNGLE YELLOW FEVER

During the year 1933 the results of an important discovery made during the course of their researches in Brazil were announced by Soper, Penna, Cardoso Serafim, Frobisher, and Pinheiro (1933) These workers for the first time drew the attention of the scientific world to a rural outbreak of yellow fever occurring in Valle do Chanaan, Espirito Santo, Brazil, during which *Aedes aegypti* could be eliminated as the insect vector Certain other mosquitoes were, however, found to be present in the locality, but the only 2 species to be viewed with suspicion were *A* (*Ochlerotatus*) *scapularis* (Rondani) and *A*. (*Taeniorhynchus*) *fluviatilis* (Lutz), of which Soper and his colleagues believed the first to be more dangerous than the second Both have been confirmed as efficient vectors of yellow fever by Whitman and Antunes (1937 a)

The results of mouse protection tests performed on the sera of several hundreds of residents in Valle do Chanaan also yielded interesting results, for, although previous epidemics had invaded that locality, the percentage of positive sera was surprisingly low. The disappearance of the disease from the district was as mysterious as the circumstances surrounding its arrival, and in explanation of its spontaneous

sued. Monkeys and mosquitoes are responsible for jungle yellow fever. Special attention has been paid to the occurrence of natural epidemics of yellow fever with high mortality affecting the Howler species of monkey which is present throughout the entire tropical region of South America Bauer (1939) has described how wild monkeys were trapped, bled for protection tests, tattooed on the ear, liberated, subsequently recaptured (some as many as 6 times) and reexamined for possible changes in antibody content. Certain species could be infected experimentally and a high enough concentration of virus appeared in the blood to infect mosquitoes, but as a group, the density of the entire monkey population in any one region seemed inadequate to sustain infection for any considerable length of time Again, at Maracaju in southern Matto Grosso, the narrow strip of forest along the streams supported such little wild life as to render animal reservoirs of infection improbable. Soper (1943) has expressed a different point of view and believes that the Howler monkey which succumbs to infection is less likely to spread infection than, for example, the Cebus monkey which circulates virus in its blood stream and so acts as a source for dissemination of virus.

At Para Brazil, in an area of jungle yellow fever, 10,121 adult mosquitoes and 1,260 larvae, representing 80 different species, were collected by Kumm and Novis (1938). No *Aedes aegypti* were found and the most common species observed to bite man by day were *Aedes nubilis*, *Psorophora ferox*, and *Haemagogus janthinomys*

Subsequent investigations on jungle yellow fever near the towns of Villavicencio and Acacias in Eastern Colombia by Bugher *et al.* (1944) have confirmed and amplified earlier etiological and epidemiological studies. No *Aedes aegypti* were found, but *Haemagogus capricornii* was found to be carrying virus on 13 occasions and *Aedes leucocelaenus* once No virus was found in any other arthropod form. Immunity tests on the sera of monkeys and the marsupial *Didelphis marsupialis* revealed that they had suffered from a recent outbreak of infection So it was concluded that jungle yellow fever can be sustained among monkeys, marsupials, or both of them, but no proof was found to indicate that these creatures constituted a reservoir of infection There was more evidence to suggest that virus survived during the dry months in *H capricornii* in which it persisted from one rainy season to another (Gast and Bates, 1945) In contrast, the behavior of yellow fever virus in *Aedes aegypti* has been found to be very different, since Davis and Shannon (1931) observed that virus was unable to survive in a fixed colony of *A. aegypti* They concluded that it seems practically certain that in nature yellow fever is not self-propagated among *Stegomyia* mosquitoes and that these insects could not maintain quantities of yellow fever among themselves without the aid of intermediate hosts More recent studies by Laemmert, Ferreira, and Taylor (1946) in Brazil forests show that virus is maintained by the primate-mosquito (*Haemagogus*) cycle Thus, the present problem is that of jungle yellow fever and the periodic introduction of infection into cities and towns by country laborers, where, should *Aedes aegypti* be present, household epidemics may ensue.

The taxonomy of the *Haemagogus* group of mosquitoes is at present being investigated In Argentina, researches showed that *Haemagogus janthinomys* (Dyar 1921) should be considered a synonym of *H. spegazzinii* (Brèthes 1912) and not a synonym of *H. spegazzinii* (Lutz) as previously suggested (Cerqueria and Boshell-Manrique, 1946) In Brazil, the geographical distribution of *H spegazzinii* and *H. uriartei* (Shannon and del Ponte) and *H capricornii* are more restricted. The Villavicencio population of mosquitoes referred to by Bates and Roca Garcia (1946 *a*) as *Haemagogus capricornii* has been identified as *Haemagogus spegazzinii* by Kumm, Osorno-Mesa and Boshell-Manrique (1946). The latter add that no less than 8 species of *Haemagogus* were found in Colombia, the commonest and most widely distributed of which was *H spegazzinii falco*

a "house disease," being contracted by individuals who visit patients in infected houses in towns or cities, and direct evidence to this effect has been furnished by Beeuwkes and Hayne (1931), who proved the presence of yellow fever virus in mosquitoes captured in African towns. Jungle yellow fever, on the other hand, is invariably contracted by persons dwelling in close proximity to forests or jungle clearings. The circumstances in which persons living amid such surroundings acquire infection enable an epidemiological distinction to be drawn between jungle yellow fever and that transmitted by *A. aegypti*. The evidence has shown that in people dwelling on the outskirts of a forest who develop the disease, infection can often be traced back to the date on which the individual entered a particular plot of jungle land. The natives of South America have themselves observed the risk of infection accompanying visits to certain fields and clearings, and Soper (1936) quotes 2 instances in which the inhabitants of a district abandoned harvesting the crop of a particular field because they knew it was dangerous to work there.

The comparative age distribution of the 2 diseases is also significant and again points to infection being acquired away from houses. *Aedes*-transmitted yellow fever tends to attack both young and old indiscriminately, but with jungle yellow fever its incidence is low among children from 1 to 4 years of age, and cases are limited to those youngsters who are sent out of doors bearing messages or food to their parents working in the forests. The highest incidence of the disease was recorded among able-bodied agricultural workers between the 2nd and 4th decades of life, also in girls of 15 to 19 years of age, just at the time when they had begun to assist their menfolk in the fields. In the case of smaller communities living in the midst of large jungles, the peculiar age distribution is absent, and here the disease appears to be endemic throughout the whole population.

Unmarried men in Colombia who are employed as itinerant woodcutters and laborers constructing roads through the forest are so frequently attacked that the illness is popularly referred to by them as "bachelor's disease." Other points of interest are that the insect vector does not enter houses built as close as 300 feet to the forests, and, furthermore, secondary cases do not develop in the homes of victims (Bauer, 1939). The next advance was made in 1938 by Shannon, Whitman, and Franca, who captured 3 species of mosquitoes at the site of an outbreak of jungle yellow fever near Rio de Janeiro and demonstrated the presence of yellow fever virus in *Aedes leucocelaenus*, *Haemagogus capricornii*, and an unidentified species of *Sabethine* mosquito. These insects breed in the holes of trees, the bromeliad and other parasitic flora of tropical forests. The distribution of *Haemagogus capricornii*, believed to be the principal diurnal vector of jungle yellow fever in Eastern Colombia, has been investigated by Bates (1944a), and found to exhibit maximum activity at midday and be relatively more numerous in the forest canopy than at ground level. *Psorophora ferox*, on the other hand, showed morning and afternoon activity. *Aedes scapularis* which breeds in grassy pools and readily transmits yellow fever under laboratory conditions, is thought to be responsible for man to man infection in rural areas and even for jungle yellow fever in flat forest zones, according to Soper (1943). At Villavicencio in Eastern Columbia, Gast and Bates (1945) correlated the incidence of human cases with seasonal fluctuation of the mosquito population, the rainy season, and the occupation of woodcutting. Thus the peak of human cases occurred in November and December, and reached their lowest ebb at the end of the dry season in March when *Haemagogus* mosquitoes were relatively scarce. Bauer (1939) mentions that the disease may be prevalent either in the tropical rain forest type of vegetation of Colombia and the Amazon or amid the shrubby grasslands and streams of southern Matto Grosso and Minas Geraes in Brazil.

The search for animal reservoirs of infection (Gilmore, 1943) among all species of wild animals inhabiting the forests of South America has been intensively pur-

fected Cebus monkeys (*Cebus versutus*), (3) by use of *Haemagogus equinus* and *H. spegazzinii* fed on marmosets. The latter insects would not breed in the laboratory. The susceptibility of primate *Leontocebus chrysomelas* in Brazil to infection was shown by Waddell and Taylor (1946) who also demonstrated presence of virus in captured C. *penicillata* on 4 separate occasions. A strain of jungle yellow fever virus was propagated for 7 cycles in *H. equinus* mosquitoes with C. *aurita* and C. *penicillata* marmosets. Under laboratory conditions *A aegypti* appeared to transmit infection more readily (by biting) than *H. equinus* (Waddell and Taylor 1947). See also Laemmert and Ferreira (1945).

Haemagogus splendens, found in Colombia, was able to transmit yellow fever under laboratory conditions to saimiri and douroucouli monkeys captured in Villavicencio. To infect mosquitoes peripheral blood of the monkey should attain a minimum concentration of 10^{-4} dilution of virus pathogenic to mice (Anderson and Osorno-Mesa, 1946).

FACTORS AFFECTING CONTROL OF YELLOW FEVER

The present situation concerning yellow fever in South America has been epitomized by Soper (1943) and Sawyer (1940) as follows·

(*a*) Absence of *Aedes aegypti* transmitted infection affecting cities, towns, villages and strictly rural areas.

(*b*) Yellow fever without *Aedes aegypti* occurring in individuals visiting the forests where the density and movement of population is so low that human cases can be incidental to an epizootic among jungle animals. Also the very rare rural type of disease suggesting a simple man-vector-man mechanism of transmission.

(*c*) Jungle transmitted yellow fever as endemic and migrating epidemic in wide areas in the interior of S. America.

(*d*) Effective methods for keeping cities free of infection by anti-*aegypti* measures.

(*e*) Active immunization of persons liable to convey infection from the forests into towns.

(*f*) Absence of recognized yellow fever of any transmission type outside S. America.

Soper (1943) states that "there is reason to believe that jungle yellow fever is the original epidemiological type and that the *aegypti*-transmitted variety is a recent and exotic development" Work in Brazil has demonstrated how *Aedes aegypti* eradication is feasible when the money, men, and authority are available for control measures. Indeed, so successful have these measures been that the *aegypti* transmitted disease, originating from previous *aegypti* transmitted yellow fever, has been absent from South America since 1934. But *aegypti* transmitted infection secondary to immediately preceding jungle yellow fever has been observed as recently as 1937 and constitutes the principle threat to control measures.

The South American continent contains vast areas of jungle yellow fever infection and the dream of yellow fever eradication from the Western Hemisphere, through anti-*aegypti* campaigns, has not been realized because jungle animals have maintained an uncontrollable reservoir of infection.

Jungle Vertebrates and Spread of Infection

Bugher, Boshell-Manrique, Roca-García, and Gilmore (1941) studied the susceptibility of certain vertebrates of the Villavicencio-Restrepo-Acacias region of Eastern Colombia to yellow fever virus. The following New World marsupials of the family Didelphidae (opossums) were shown susceptible to the virus of yellow fever and some developed antibodies after recovery, suggesting that they may be vital links in the complex epidemiological picture of jungle yellow fever in Colombia. Genus 1, *Didelphis* (Linnaeus), 1758 Large, common opossum, "chucha," "fara," "runcha" (Colombia). Species tested were *Didelphis marsupialis* and *Didelphis paraguayensis*. Genus 2, *Philander* (= *Metachirops* auct) Brisson, 1762. Gray masked opossum; "chucha real" (Colombia). According to Laemmert (1946) *Didelphis marsupialis*, *Didelphis paraguayensis*, and *Metachirops opossum* are relatively insusceptible to infection Genus 3, *Metachirus* (Burmeister, 1854). "Chucha real" (Colombia) closely resembling *Philander* save for longer tail and browner coloration *Metachirus nudicaudatus*. (Geoffroy, 1803), was examined. Laemmert (1946) also reported that *Metachirus nudicaudatus* and *Marmosa incana* could be readily infected. *Marmosa cinera* was partially susceptible. Genus 4, *Chironectes*, the water opossum, and Genus 5, *Lutreolina*, the weasel opossum, were not tested Genus 6 *Caluromys* (=*Philander* auct) Allen, 1900. Brown or woolly opossum *Caluromys* (*Mallodelphis*) *laniger*. Genus 7, *Marmosa* (Gray, 1821). Long, bare-tailed murine opossum. "Chucha raton" (eastern Colombia). Common to Central and South America

At Villavicencio, the *Saimiri sciureus caquetensis* monkey also referred to as the "squirrel" monkey in English, and "mico titi" in Colombia, was proved to be highly susceptible to yellow fever virus infection The maximum titer of virus in circulating blood was found to reach a dilution of 1 1,000,000 and about 33 per cent succumbed to infection (Bates, 1944 *b*). The brown masked opossum *Metachirus nudicaudatus* was found susceptible to intramuscular injection, and a Colombian strain of yellow fever virus was maintained for 10 consecutive passages in this animal. The serum of the latter was toxic to mice by intracerebral inoculation The gray masked opossum, *Metachirops opossum*, was resistant (Bates, 1944 *c*) Marmosets *Callithrix leucocephala* (E Geoff), *Callithrix jacchus* (L) and the lion marmoset *Leontocebus rosalia* have been shown to develop fatal infections (Laemmert, 1944) The cane rat, *Zygodontomys stella*, indigenous to Villavicencio, is also susceptible to intracerebral inoculation with yellow fever virus (Bates and Weir, 1944). See also Laemmert (1946) for susceptibility of marsupiala to different strains of yellow fever virus

Cyclical Transmission Experiments

Subsequent studies in Colombia by Bates and Roca-García (1946 *a*) on insects and mammals indigenous to those lands, have shown that the virus of sylvatic yellow fever could be maintained for one year of 14 cycles, by alternate passage through insects and mammals.

The infective cycle was readily demonstrated employing saimiri or douroucouli (*Aotus trivirgatus*) monkeys said to be the only species of monkey prevalent in Eastern Colombia where yellow fever is endemic (Bates and Roca-García, 1945). Also the marmoset *Oedipomidas oedipus* Virus circulates in the blood of monkeys for 7 to 8 days and in *Haemagogus capricornii* for the duration of its life The mosquito, therefore, appears to be a permanent reservoir of yellow fever virus In the Ilhéos region of Balna, Brazil, yellow fever virus has been isolated both from the wild marmoset (*Callithrix penicillata*) and the mosquito *Haemagogus spegazzinii* (Laemmert, Ferreira and Taylor, 1946) and cyclical transmission was also demonstrated in 3 other ways by Waddell and Taylor (1945) (1) by feeding *Aedes aegypti* on infected *Callithrix aurita*, (2) by feeding *Aedes aegypti* on in-

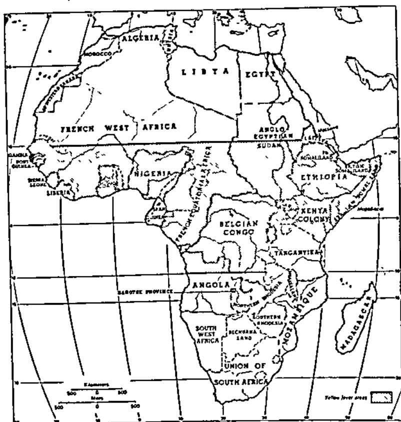


Fig. 36 African yellow fever area, delineated by Expert Commission on Quarantine Minor changes have since been made in Eritrea and N Rhodesia

(Reproduced from *Epidem Inform Bull*, 1945, *1*, 689)

no evidence of infection in Tanganyika and Zanzibar; Abyssinia is yet to be surveyed

After a lapse of 10 years, active work was renewed at the Yellow Fever Research Institute, Lagos, Nigeria (1945). Only one case was reported in 1942, but there was reason to suspect that the disease had been widespread among Africans throughout Nigeria south of latitude 10° N. Several thousands of cases are said to have occurred and the difficulty of recognizing infection in the African was stressed. Europeans appear to have escaped the disease, in all probability due to the efficacy of vaccination.

Does Jungle Yellow Fever Exist in Africa?

There is no absolute proof that the jungle variety of disease (analogous to that occurring in S America) exists in Africa. More than 150 years ago Lind (1792), a surgeon with the Royal Navy, reported that when the sloop *H.M.S Merlin* lay at anchor in the Gambia River in August, 1768, only those sailors who went into the forests to fell wood contracted yellow fever, whereas men remaining on board escaped.

Recent accounts suggest that hitherto unrecognized jungle yellow fever may be prevalent in Africa.

CHAPTER XLI

YELLOW FEVER IN AFRICA

From 1810 to 1878 Hirsch (1883)[1] reported successive epidemics of yellow fever in Senegambia, Sierra Leone, the Gold Coast, Teneriffe, Las Palmas, The Cape Verde and Ascension Islands (see also Boyce, 1910). In subsequent years the case incidence has declined, and in 1936 only 11 cases were reported from Dahomey, Guinea, Ivory Coast, Niger Territory, and Senegal, with 9 cases from the British Colonies including Sudan, the Gold Coast, and Nigeria. In 1940 an extensive epidemic of yellow fever occurred in Nuba Mountains region of Anglo-Egyptian Sudan (Kirk, 1941). In Western Uganda an important focus of disease was discovered in that part of the valley of the Semliki River to the west of the Ruwenzori Mountains which embraces the most easterly reaches of the Ituri forest of the Bwamba country, by Mahaffy, Smithburn, Jacobs, and Gillett (1942).

For official information of the endemic areas of yellow fever the reader should consult the periodical Epidemiological Information Bulletin published by the United Nations Relief and Rehabilitation Health Division. The last announcement delineated the African area of infection as follows

From the mouth of the River Senegal along that river eastward to the 15° N. parallel of latitude, thence eastward along that parallel to the eastern border of the Anglo-Egyptian Sudan, thence northward along the northwestern boundary of Eritrea to the Red Sea Coast, thence southward along the eastern coast of Africa to the southern boundary of the Protectorate of Kenya, thence westward along that boundary and the southern boundary of Kenya Colony to its junction with the southern border of the Uganda Protectorate, and thence along this and the eastern border of the Belgian Congo to the 10° S parallel of latitude, thence westward along that parallel to the west coast of Africa, thence northward along the west coast of Africa to the mouth of the River Senegal including the islands of the Gulf of Guinea The port of Massawa in Eritrea is excluded (de Paula Souza, 1945)

The African area can be divided into 2 parts. The western area extending from Nigeria to Angola where urban outbreaks are due to *A aegypti* (Beeuwkes *et al*, 1934) The eastern area lies between the latitudes of 3° and 8° N. extending from the eastern French Cameroons across French Equatorial Africa, overlapping northern Belgian Congo and into Anglo-Egyptian Sudan as far as Rumbeck. Within this area Sawyer and Whitman (1936) have reported high prevalence of immunity among children and adults North and south of the above territories there is a diminishing incidence of immunity Tests suggest the absence of immunity in southeastern Belgian Congo and Angola The Barotze Province of N. Rhodesia appears to be an island of infection surrounded by healthy country. De Paula Souza (1945) states that "the danger of the disease spreading eastward to seaports on the Indian Ocean demands much greater caution in determining the marginal areas of infection in Africa than doing so in S America." Thus it is safer to extend the area of quarantine to the Indian Ocean and Italian Somaliland The distribution of immunity to yellow fever in central and East Africa is described by Mahaffy, Smithburn, and Hughes (1946) They report that yellow fever has occurred recently in Belgian Congo, Anglo-Egyptian Sudan, Eritrea, Somalia, Kenya, and Northern Rhodesia Positive mouse protection tests were returned from as far east as Eritrea on the Red Sea, and to the south from Balawao in N Rhodesia. There has been

[1] References are appended at the conclusion of Ch XLIII, p. 511 et seq

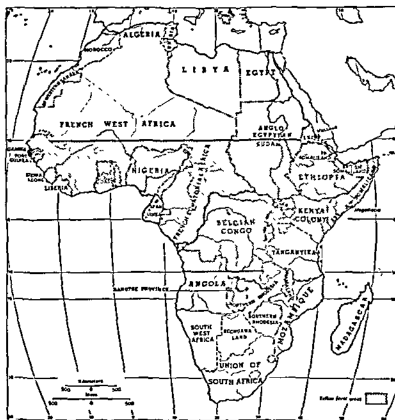


FIG 36 African yellow fever area, delineated by Expert Commission on Quarantine. Minor changes have since been made in Eritrea and N. Rhodesia

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Recent accounts suggest that hitherto unrecognized jungle yellow fever may be prevalent in Africa.

Smithburn and Haddow (1946) isolated virus from *Aedes simpsoni* caught in 1942 in a forest plantation in western Uganda 11 months after mass immunization of the human population Virus was again isolated in 1944, from a lot of 80 *Aedes* mosquitoes including 12 different species, but not including *Aedes aegypti* or *A simpsoni*. The latter were collected in the uninhabited Semliki Forest, indicating an extrahuman cycle of virus activity, involving a forest vector. Sawyer (1940) holds that both S. America as well as Africa contain endemic regions from which yellow fever escapes from time to time to start urban *aegypti* transmitted epidemics and to spread through the paths of commerce.

Data suggested that human infection was associated with contact with the forest, and Mahaffy, Smithburn, Jacobs, and Gillett (1942) showed that in the Bwamba uninhabited forest region there were 4 species of mosquitoes known to be potential vectors of yellow fever, namely *Eretmopodites chrysogaster* (Graham), *Aedes (Stegomyia) simpsoni* (Theobald), *Taeniorhyncus (Mansonioides) africanus* (Theobald), *Aedes (Stegomyia) africanus* (Theobald). The last named species is by far the most prevalent, it breeds in plant axils and is found in bananas, pineapples, dracaena and colocasia, a widely cultivated food plant. It is not found inside houses but in their immediate vicinity. See also Haddow, Gillett, and Heighton (1947). The abundance, distribution, and feeding habits of some West African mosquitoes has been described by Kerr (1933).

Mahaffy *et al.* (1942) have summarized the position in Africa as follows "There are many gaps in our knowledge of the epidemiology of yellow fever in Africa and the whole problem requires further intensive study The possibility of the virus being able to maintain itself in sparsely populated or even uninhabited areas cannot be excluded. It may be that there are reservoirs of infection as yet unknown. The lore of yellow fever epidemiology associates forests and rainfall with outbreaks of the disease in a nonurban population. The experience in Africa strongly supports the importance of both these factors, but it has been found that some areas with forests and adequate rainfall manifest much less activity than others quite similar in these respects. The factors responsible for these variations in the virus in different areas in Africa are at present not known." According to Smithburn and Haddow (1946), in Bwamba the red tail monkey *Cercopithecus nictitans mpangae* Matschie, which frequently raids banana plantations may establish an extra human cycle of infection with *Aedes africanus* occurring in the forest canopy. At Gede, a settlement 65 miles north of Mambasa 3 miles from the sea, 3 species of acrodendrophilous mosquitoes, namely, *Aedes (Diceromyia) adersi*, *taylori*, and *furcifer*, have been suspected as jungle vectors of yellow fever (Bailey, 1947).

Some conception of the magnitude of the task confronting the investigator of jungle yellow fever in Africa is well illustrated in the complexity of the prevailing mosquito problem in Anglo-Egyptian Sudan Lewis (1947), has reported the occurrence of 140 known species, divisible into 3 categories (*a*) those which bite man readily (39 species), (*b*) those common to certain areas but which seldom or never attack man (18 species), and (*c*) a group comprising 9 species of which more information is sought

There exist 12 species capable of transmitting yellow fever by bite and these are *Taeniorhyncus africanus*, *Aedes aegypti*, *A aegypti* var *queenslandensis*, *A. simpsoni var lilii*, *A metallicus*; *A africanus*, *A leuteocephalus*; *A. vittatus*, *A stokesi*; *A taylori*; *Eretmopodites chrysogaster*, and *Culex fatigans* Equally important in the chain of possible jungle reservoirs of infection are 2 species *Taeniorhyncus uniformis* and *Aedes lineatopennis* which remain infective throughout life but are incapable of transmitting infection by biting as far as can be determined *Anopheles gambiae* and *Aedes apicoargenteus* only retain virus for a short time The African grivet monkey, *Cercopithecus aethiops centralis* Neuman, can, in a proportion of animals, be infected with yellow fever virus, and circulate in high

concentration of virus in its peripheral blood stream (Hughes, 1943). The latter has explained how the lower primates may serve as mobile distributors of virus, especially if the illness produced is not severe, by carrying infection from the host to an area infested with domestic mosquitoes In this manner primates may serve as a link in the chain of events culminating in human infection. It is possible that the palm civet cat *Nandina binotata arborea* may function as a reservoir of virus, the galagos and pottos lemurs are probably of little importance in most areas (Rockefeller Report, 1946)

CHAPTER XLII

PROPERTIES OF THE VIRUS OF YELLOW FEVER

Filtrability

REED and Carroll (1902)[1] and Reed, Carroll, Agramonte, and Lazear (1911) were the first workers to demonstrate that the causal agent of yellow fever was capable of passing through the pores of a Chamberland F filter which were too small for ordinary bacteria to traverse. This finding was soon confirmed by Marchoux, Salimbeni, and Simond (1903) and Rosenau, Parker, Francis, and Beyer (1905). The experiments, however, could be performed only on a limited scale, as the work necessitated the use of a human volunteer on each occasion on which it was desired to test the pathogenicity of a filtrate More extensive filtration experiments were possible after Stokes, Bauer, and Hudson (1928–38) discovered the susceptibility of the *M. rhesus* monkey to infection. After this important observation, Stokes and his colleagues showed that the virus contained in infected monkeys' blood was able to pass the Berkefeld V and N candles as well as the Seitz (E K) asbestos disk filter, at a negative pressure of 400 mm. of mercury. The Berkefeld W candle, on the other hand, retained the agent even when a pressure of 720 mm. was applied Attempts to demonstrate the filtrability of the virus present in infected mosquitoes were also made, but these proved unsuccessful. Apparently, either the agent was not present in a small enough phase in the insect to be filtrable, or else there was insufficient amount to compensate for loss of virus due to adsorption during filtration. Bauer and Mahaffy (1930 *a*) passed the virus through the Berkefeld V, N, and W candles, as well as through the Pasteur-Chamberland L_{11} bougie The presence of serum assisted its filtrability, and the virus survived the pH range 4 8 to 7 8 (Frobisher, 1931 *c*) Kotter and van den Berghe (1935) filtered a 1/1,000 dilution of virulent mouse brain virus through the EK asbestos disk of a Seitz filter.

Size

Findlay and Broom (1933) filtered the virus through graded collodion membranes and found its size to measure from 17 to 28 mμ in diameter. Their work was confirmed later by Bauer and Hughes (1935), who used similar ultrafiltration methods and also concluded that it was exceedingly small in size and measured less than 50 mμ in dimensions The latter investigators also established the point that the size of the virus was not altered as a result of frequent passage through monkeys or intracerebral inoculation of mice For these researches 2 strains of agent were employed, the one called the "Asibi" strain which was originally isolated by Stokes, Bauer, and Hudson in 1927, and subsequently maintained virulent by frequent passage through rhesus monkeys, and the other, a virus isolated by Mathis, Sellards, and Laigret (1928) at Dakar, which had been adapted to mice by Theiler (1930) and had been passaged over 150 times intracerebrally in mice

Preservation of Yellow Fever Virus

Virus could be regularly passaged in monkeys or mice using infected liver or brain tissue respectively for inoculation In the frozen state the material retains its virulence for several months, and Sellards and Hindle used this method for transporting the "French" strain from Senegal to America

Sawyer, Lloyd, and Kitchen (1929) reported that it died out in citrated or clotted blood if kept in a refrigerator, and rarely survived for longer than 35 days

1 References are appended at the conclusion of Ch XLIII, p. 511 et seq

if preserved by this method. It remained active in 50 per cent. glycerol at 0° C. for 100 days, but underwent deterioration thereafter. The most successful method, however, was to desiccate infected blood or liver tissue by drying it from the frozen state *in vacuo*, after which it was preserved for 2 years. Hindle (1929, 1930, 1930 *b*) and Hudson and Klotz (1928) obtained similar results previously (see also Sellards and Hindle, 1928. See also p 504).

Action of Physical and Chemical Agents

Heat.

The virus is destroyed by heating infective material to 65° C. for 10 minutes (Hindle, 1930 *a*), and both neurotropic and viscerotropic varieties of yellow fever virus possess the same degree of resistance to heat (Frobisher, 1933).

Chemical agents.

Both the neurotropic and viscerotropic strains of yellow fever virus appear to be equally susceptible to the action of certain chemical substances. Viscerotropic virus in cell-free serum was inactivated by exposure for 30 minutes at 30° C. to 6 7 per cent. ethyl alcohol and 0 3 per cent. phenol. Tests with eosin, mercuric chloride, and formalin necessitated the use of monkeys as test animals, owing to the toxicity of these substances to mice (Frobisher, 1930, 1933). A dilution of 1/1,000 formaldehyde rapidly kills the virus at room temperature and even at 0° C. its virulence is destroyed in 48 hours It resists a mixture of 60 per cent. glycerol and 0 5 per cent phenol for 5 days at 0° C, but at 16° C. it is killed within 48 hours (Hindle, 1929) Yellow fever virus is not affected by sulfapyridine or sulfathiazole (Koprowski and Lennette 1944 *a*, 1944 *b*).

Photodynamic action of methylene blue.

Findlay (1934 *a*) has demonstrated that both the viscerotropic and neurotropic strains of yellow fever virus may be inactivated by treatment with a 1 in 100,000 dilution of methylene blue, and subsequent exposure to a pointolite source of light for 20 minutes. Virus killed by this method also loses its antigenic properties According to Frobisher (1933), neurotropic yellow fever virus is more resistant to the action of 0 3 per cent methylene blue than the viscerotropic variety.

The effect of acidity and alkalinity.

Hindle and Findlay (1930) showed that viscerotropic virus was destroyed in acid media of pH 3 0 to 4 0 Frobisher (1933) found that the neurotropic virus present in serum suspensions resisted 3 hours' exposure to acidities and alkalinities ranging from pH 5 7 to pH 9 2, but at pH 5.0 it was inactivated after 3 hours (Frobisher, 1931 *b*)

Electrical charge of the virus.

Hindle and Findlay (1930) state that the viscerotropic virus possesses a negative charge between the range pH 5 2 to pH 7.0. The behavior of neurotropic and viscerotropic strains of yellow fever virus when placed in the electric field has been investigated by Frobisher (1933), who found that the neurovirus exhibited 2 broad iso-electric zones, the one in the acid range below pH 6 9 and the other in the alkaline range above pH 7 2 In the case of viscerotropic virus, its iso-electric point was found to occupy the range between pH 6.9 and pH 7 2 The electrical charge of the virus neutralizing antibodies present in yellow fever antiserum has been investigated by Frobisher (1931 *b*) and found to be electronegative at pH 7.4

Adsorption, elution, and purification of yellow fever virus.

Hughes (1934) used the following technique

The brains of 10 infected mice were removed, emulsified in 100 c c. of distilled

water, centrifuged for 30 minutes at high speed, and the supernatant fluid passed through a Berkefeld N filter. To 5 c.c. of the filtrate, 10 c.c. of a glycine-acetate buffer of the desired pH and 5 c.c. of a stock 40 per cent. suspension of kaolin in distilled water were added. The final pH of the mixture was determined electrometrically. The mixtures were allowed to stand at room temperature for 6 hours, then centrifuged, the supernatant fluid being passed through a Berkefeld N filter and tested for the presence of virus The protein content of the filtrate was estimated by Kjeldahl's method and an appropriate correction made for the amount of nitrogen in the buffer. Results showed that the virus is completely adsorbed throughout its range of survival from pH 5.1 to 9.8, but the amount of protein adsorbed decreases with increasing pH values. Thus the virus could be completely adsorbed out of solutions of low protein content (below 0.5 per cent protein) by treatment with 40 per cent. kaolin for 15 minutes.

Elution The optimum concentration of eluent required for liberating the maximum quantity of virus and at the same time releasing the smallest amount of associated protein was also experimentally determined Thus, treatment with N/100 NH_4OH freed 80 per cent. of the original virus content with 3 per cent of the original protein

Resistance to putrefaction.

Bauer (1931) reports that the virus is capable of surviving in the tissues of infected monkeys even if the animals have been dead for a long time, and their organs are in an advanced state of decomposition

Reaction to compression.

The effect of high pressures on yellow fever virus has been studied by Bisset, Nicolau, and Macheboeuf (1935), these workers subjected infective material to 1,000, 2,000, and 3,000 atmospheres of pressure and found that material thus treated, when inoculated into animals, caused death a few days later than usual.

Cultivation of Yellow Fever Virus

Methods similar to those employed by Rivers (1931) and Rivers and Ward (1933) have been used for this purpose by Lloyd, Theiler, and Ricci (1936) and Theiler and Smith (1937) The latter first passaged the "Asibi" strain of virus through 240 subcultures in a medium consisting of minced mouse embryonic tissue and 10 per cent normal monkey serum in Tyrode's solution over a period of 3 years At the end of this period the virus was designated the "17E" strain and was sufficiently attenuated for human prophylactic vaccination

After the 18th passage in the above medium, a separate strain, which was duly named the "17D" strain, was evolved by propagating the virus for 58 subcultures in a medium consisting of minced whole chicken embryo, followed by 160 further passages in chick embryo medium prepared from decerebrated and demyelinated embryos. A 3rd strain of virus was derived from the strain "17E," after its 27th subculture, by propagating it serially 70 times through another medium composed of minced adult mouse testis, followed by 90 further passages in which adult guinea-pig testis was substituted for mouse testis The latter was called the "17AT" strain.

Throughout the whole experiment cultures were incubated at 37° C and subinoculated every 3 to 4 days At each stage cultures were centrifuged, the supernatant fluid tested for infectivity, and a quantity of the material preserved for future use by desiccating it from the frozen state.

Haagen and Theiler (1932) successfully cultivated the virus in tissue cultures consisting of 9- to 10-day-old minced chicken embryos, or else in media prepared from rabbit or guinea-pig kidney or testicle tissue. After 22 passages had been carried out, the culture was tested for pathogenicity and found to produce encephalitis

when injected intracerebrally in a mouse In a subsequent publication Haagen (1933) reported that he was able to maintain yellow fever virus in tissue cultures for more than 100 passages without inducing any change in the properties of the virus The virus was readily neutralized *in vitro* by the addition of immune serum, but once it had entered the tissue cells it was capable of withstanding the action of concentrated immune serum

Infection of mouse embryos *in utero*.

During the course of their experiments, Smith and Theiler (1937) inoculated mouse embryos *in utero* with yellow fever virus. Mice (Swiss strain) were inoculated about the 13th day of pregnancy, 0 02 c c. of virus material being introduced into the uterus with a 26-gage needle through an incision made into the abdominal cavity Four days later the embryos were removed and tested for presence of virus, which appeared in maximum concentration in the brain of the embryo Unmodified strains of virus were also adapted to cultivation *in vitro* in a medium consisting of minced mouse embryo brain tissue in Tyrode's solution containing 10 per cent. monkey serum Later, whole mouse embryo was substituted for the above There was also some evidence to suggest that the neurotropism of the virus for mice could be increased by prolonged cultivation in mouse embryo brain medium Attempts to employ monkey tissues were unsuccessful

Growth of virus in tumor-bearing mice.

Findlay and MacCallum (1937 *b*) grew the virus in the cells of an actively growing mouse carcinoma The tumors were inoculated with a strain of neurotropic virus which was propagated for 50 passages in this manner without loss of pathogenicity. A pantropic strain of virus, which was highly pathogenic for monkeys and hedgehogs, was passaged 65 times through mouse carcinomata and showed considerable loss of virulence, without increase in neurotropism, for these animals after such treatment Findlay and MacCallum also made the interesting observation that yellow fever virus appeared to exhibit a special affinity for neoplastic cells, since it tended to become localized in these tissues following inoculation by subcutaneous or intraperitoneal injection

Cultivation on the chorio-allantoic membrane.

Recent experiments have shown that yellow fever virus may be cultivated in a manner analogous to that of other viruses on this medium Elmendorf and Smith (1937) reported that they were able to propagate unmodified as well as tissue-culture-adapted strains of virus on the developing chicken embryo Altogether 18 subcultures were made, and successive embryos were infected by inoculating them either with the fluid derived from the surface of the chorio-allantoic membrane or else with the minced embryo Similar results have been reported by Jadin (1937)

INSECT VECTORS OF YELLOW FEVER VIRUS

Mosquitoes

The early work of Reed (1902), Reed *et al* (1911), and Marchoux and Simond (1906 *a*) necessitated the use of human volunteers to determine whether insects which had fed on human cases contained the virus or not The experiments answered their purpose for the time being, as they conclusively proved the rôle of *Aedes* as a vector of infection, but were unsatisfactory because the need for human experimental subjects limited the size of tests, and, moreover, the presence of negative results signified nothing, because the individuals used may have been immune to infection

After Stokes and his coworkers discovered the susceptibility of the rhesus

monkey to infection, a great impetus was given to the further study of insect vectors of yellow fever. Table 17 gives a summary of some of the tests which have been carried out with various insects. The general practice of most workers has been to infect the mosquitoes by allowing them to bite a human patient or an infected monkey, or, less frequently, by permitting them to feed on a specially prepared medium containing infected blood. The infected mosquitoes are then tested for the presence of virus by inducing them to bite a normal rhesus monkey. In the event of the insects refusing to feed upon the animal, or the tests being negative, they are then emulsified in saline and injected into the monkey with a hypodermic needle. The results tabulated on p. 489 are by no means comprehensive, but indicate some of the many tests made. Under laboratory conditions, it is apparent that yellow fever can be transmitted by a great many insect vectors other than the principal one, *Aedes aegypti*, but to what extent this occurs in nature is unknown Since Soper (1936) reported that the virus can be transmitted from one monkey to another in the laboratory by mosquitoes of the genus *Haemagogus*, investigations have been resumed afresh from a different angle. Less emphasis has been paid to the search for the existence of simple man-mosquito cycles of infection in urban areas and the pendulum has swung in the direction of a hunt for jungle vertebrate reservoirs of yellow fever and forest mosquito vectors capable of perpetuating the infective cycle in forest zones uninhabited by man. Some of the earlier work on insect vectors may have to be extended in the light of current developments Table 17 contains a record of earlier data.

A summary of previous work on mosquitoes.

The rôle of various species in the propagation of yellow fever is conveniently tabulated in Table 17

BEHAVIOR OF VIRUS IN AEDES AND HAEMAGOGUS MOSQUITOES

The evidence shows that yellow fever virus neither passes through a developmental phase in the mosquito nor does it multiply appreciably in its body. Regarding the first point, work on the growth of yellow fever virus in tissue cultures proves that its passage through the insect vector is not an essential feature of its life history. Secondly, there is no evidence to show that the virus multiplies greatly during the period of its sojourn in the mosquito. Gay and Sellards (1927) measured the infectivity of the virus in insects over a period of 3 weeks, and found that, following an initial increase after feeding, it subsequently remained at a constant level and could be demonstrated in the head, thorax, abdomen, legs, ovaries, salivary glands, and the mid and hind gut of the insect (see also Davis and Shannon, 1930).

Davis, Frobisher, and Lloyd (1933), using Aedes mosquitoes as vectors and rhesus monkeys as test animals, found that the average mosquito, immediately after engorgment, had ingested from 1 to 2 million lethal doses of virus A fortnight later there was a slight decrease in its amount followed by a rise, attributable to an increase of extracellular virus At no time, however, was the quantity of virus titrated equivalent to that demonstrated in the insect immediately after feeding Findings contrary to these have been recorded by Whitman (1937), who titrated the amount of *virus present in A aegypti at different times after infection*, and found that 38 days after it had fed, the titer greatly exceeded the original amount of virus ingested.

At first sight these two statements appear to be conflicting but, as we shall explain, it is quite possible that the virus may multiply greatly in some *Aedes*, whereas in others it may only do so to a lesser extent, and so both workers may be correct According to one explanation advanced by Davis, Frobisher, and Lloyd

TABLE 17

AFRICAN MOSQUITOES

Species	Results of Biting Test: Positive (+) Negative (o)	Results of Biting Test: Number of days after meal on infected blood	Results of Injection of Emulsion: Positive (+) Negative (o)	Results of Injection of Emulsion: Greatest number of days positive	Authority
Aec ... *na) aegypti* (Linn.)	+	8	—[1]	—	Philip (1930)
Aec ... *ia) aegypti* (Linn.) pale form	+	12	—	—	Lewis, Hughes, and Mahaffy (1942)
Aec ... *ia) africanus* (Theo.)	+	12	—	—	Philip (1929)
Aec ... *rphus) apicoannulatus* (... imed Stokesi Edw.)	+	19	—	—	Philip (1929)
Aec ... *ia) apicoargenteus*	o	—	o	—	Bauer (1928)
Aec ... *rphus) irritans* (Theo.)	o	—	+	51	Kerr (1932)
Aec ... *iella) lineatopennis* (Ludl.)	o	—	+	?	Kerr (1933)
Aec ... *) longipalpis* (Grunb.)	o[2]	—	—	—	Bauer (1928)
Aec ... *ia) luteocephalus* (Newst.)	+	15	—	—	Bauer (1928)
Aec ... *ia) metallicus*	+	10	—	—	Lewis, Hughes, and Mahaffy (1942)
Aec ... *rphus) nigricephalus* (Theo.)	o	—	+	?	Philip (1930 *a*)
Aea ... *ella) punctocostalis* (Theo.)	o	—	+	?	Philip (1930 *a*)
Aea ... *ia) simpsoni* (Theo.)	+	19	—	—	Philip (1929), Smithburn, and Haddow (1946)
Aea- ... *rphus) stokesi* (Edw.) ... *apicoannulatus* (Edw.)	+	19	—	—	Bauer (1929)
Aea ... *yia) taylori*	+	12	—	—	Lewis, Hughes, and Mahaffy (1942)
Aed ... *ia) vittatus* (Bigot) (... heo.)	+	11	—	—	Philip (1929)
Aed ... *) woelmani* (Theo.)	o[2]	—	—	—	Bauer (1928)
Anc ... *omyia) gambiae* (Giles) ((... W.)	o	—	o	—	Philip (1930)
Cule ... *yia) nebulosus* (Theo.)	o[2]	—	—	—	Bauer (1928)
Cule ... *halassius* (Theo.)	+	27	—	—	Kerr (1932)
Fret ... *hrysogaster* (Graham)	+	16	+	24	Bauer (1928)
Tae ... *(Mansonioides) africanus* (T ...	+	16	+	12	Philip (1930)

[1] The sign (—) indicates no result recorded

[2] These species refused to feed on monkeys

monkey to infection, a great impetus was given to the further study of insect vectors of yellow fever. Table 17 gives a summary of some of the tests which have been carried out with various insects. The general practice of most workers has been to infect the mosquitoes by allowing them to bite a human patient or an infected monkey, or, less frequently, by permitting them to feed on a specially prepared medium containing infected blood. The infected mosquitoes are then tested for the presence of virus by inducing them to bite a normal rhesus monkey. In the event of the insects refusing to feed upon the animal, or the tests being negative, they are then emulsified in saline and injected into the monkey with a hypodermic needle. The results tabulated on p. 489 are by no means comprehensive, but indicate some of the many tests made. Under laboratory conditions, it is apparent that yellow fever can be transmitted by a great many insect vectors other than the principal one, *Aedes aegypti*, but to what extent this occurs in nature is unknown. Since Soper (1936) reported that the virus can be transmitted from one monkey to another in the laboratory by mosquitoes of the genus *Haemagogus*, investigations have been resumed afresh from a different angle. Less emphasis has been paid to the search for the existence of simple man-mosquito cycles of infection in urban areas and the pendulum has swung in the direction of a hunt for jungle vertebrate reservoirs of yellow fever and forest mosquito vectors capable of perpetuating the infective cycle in forest zones uninhabited by man. Some of the earlier work on insect vectors may have to be extended in the light of current developments. Table 17 contains a record of earlier data.

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TABLE 17—Continued

S American Mosquitoes—Continued

Species	Results of Biting Test: Positive (+) Negative (o)	Results of Biting Test: Number of days after meal on infected blood	Results of Injection of Emulsion: Positive (+) Negative (o)	Results of Injection of Emulsion: Greatest number of days positive	Authority
Joblotia digitata (Rondani)	o	—	o	—	Davis and Shannon (1931 *a*), Kumm and Frobisher (1932)
urhami (Theo)	o	—	o	—	Davis and Shannon (1931)
(*Rhynchotaenia*) *albicosta* sû 1908)	o	—	+	14	Davis and Shannon (1931 *a*), Whitman and Antunes (1937)
(*Mansonia*) *chrysonotum* sû)	o	—	+	10	Davis and Shannon (1931 *a*)
(*Rhynchotaenia*) *chrysonoturn* su 1922)	o	—	+	23	Whitman and Antunes (1937), Davis and Shannon (1931 *a*)
(*Rhynchotaenia*) *fasciolata* Arribálzaga 1891)	o	—	+	16	Whitman and Antunes (1937), Davis and Shannon (1931)
(*Rhynchotaenia*) *justamansonia* - 1907)	o	—	+	14–22	Whitman and Antunes (1937)
(*Mansonia*) *titillans* (Walker)	o	—	+	30	Kumm and Frobisher (1932)
r (*Psorophora*) *ciliata* (Fabricius)	o	—	o	—	Marchoux and Simond (1906 *a*)
i (*Grabhamia*) *cingulata* us)	—	—	+	14	Davis and Shannon (1931 *a*)
i (*Janthinosoma*) *ferox* ldt 1820)	o	—	+	35	Whitman and Antunes (1937 *a*), Davis and Shannon (1931 *a*)
i (*Janthinosoma*) *posticata* Same species as *ferox*	o	—	o	—	Marchoux and Simond (1906 *a*)
(*Pentemyia*) *bromeliarum* nd Knab)	o	—	o	—	Davis and Shannon (1931)
(*Wyeomyia*) *oblita* (Theo)	o	—	o	—	Davis and Shannon (1931)

TABLE 17—*Continued*

S American Mosquitoes

Species	Results of Biting Test: Positive (+) Negative (o)	Results of Biting Test: Number of days after meal on infected blood	Results of Injection of Emulsion: Positive (+) Negative (o)	Results of Injection of Emulsion: Greatest number of days positive	Authority
Aedes (Taeniorhynchus) fluviatilis (Lutz)	+	16	—	—	Davis and Shannon (1931)
Aedes (Taeniorhynchus) fluviatilis (Lutz 1904)	+	16–26	—	—	Whitman and Antunes (1937 *a*)
Aedes (Howardina) fulvithorax (Lutz 1904)	o	—	o	—	Davis and Shannon (1931 *a*) and Whitman and Antunes (1937)
Aedes (Ochlerotatus) hastatus (Dyar)	o	—	—	—	Davis and Shannon (1929)
Aedes (Ochlerotatus) nubilus (Theob 1903)	o	—	+	19–27	Whitman and Antunes (1937)
Aedes (Ochlerotatus) scapularis (Rondani 1848)	+	16–23	+	30	Whitman and Antunes (1937), Davis and Shannon (1929)
Aedes (Ochlerotatus) serratus (Theo)	o	—	+	31	Davis and Shannon (1929)
Aedes (Taeniorhynchus) taeniorhynchus (Wiedermann 1821)	+	19	+	24	Davis and Shannon (1931), Whitman and Antunes (1937)
Aedes (Finlaya) terrens (Walker 1856)	o	—	+	17–38	Davis and Shannon (1931), Whitman and Antunes (1937)
Anopheles (Nyssorhynchus) albitarsis (Lynch Arribalzaga)					
Anopheles (Nyssorhynchus) tarsimaculatus (Goeldi)	o	—	o	—	Davis and Shannon (1931 *a*)
Culex corniger (Theo)	o	—	o	—	Davis and Shannon (1931 *a*)
Culex coronator (Dyar and Knab)	o	—	o	—	Kumm and Frobisher (1932)
Culex nigripalpus	o	—	o	—	Kumm and Frobisher (1932)
Culex (Culex) quinquefasciatus (Say) (syn. fatigans)	o	—	+	7–16	Davis, see Whitman and Antunes (1937)
Culex surinamensis (Dyar)	o	—	o(?)	—	Davis and Shannon (1929)
Haemagogus capricornii	o	—	o	—	Kumm and Frobisher (1932)
Haemagogus equinus	+	10–28	—	—	Bates and Roca-Garcia (1945–6 *a*)
Haemagogus janthinomys (Dyar) (syn spegazzinii)	+	21	—	—	Waddell and Taylor (1945)
Haemagogus spegazzinii (Brèthes)	+	14	+	16	Antunes and Whitman (1937)
	—	—	+	Naturally infected	Laemmert, Ferreira, and Taylor (1946)
Haemagogus splendens	+	14–16	—	—	Anderson and Osorno-Mesa (1946)
Haemagogus uriartei (Shannon and Del Ponte)	o	—	+	16	Antunes and Whitman (1937)

(1933), the extrinsic incubation period in the mosquito is the time occupied by the virus in its migration to the salivary glands, and does not represent the interval required for its multiplication Sellards (1935 *a*) has taken the opposite view and maintains that, following a meal of infected blood, much of the virus present in it is destroyed by the tissues of the mosquito, and this would account for the fall in infectivity observed in the days immediately succeeding the intake of blood. The surviving virus then commences to multiply in the mosquito so that, at the end of the incubation period, the bite of the insect proves infective. Sellards points out that the behavior of the virus in the mosquito is analogous to that which is known to occur in the case of protozoal blood parasites, after they have been ingested by certain blood-sucking arthropod vectors If, therefore, Sellards's interpretation were to be accepted, it would explain the divergence of results recorded, since the degree of infectivity of a mosquito at the end of the incubation period is a matter of chance, depending on the extent to which the virus has flourished in the tissues of that particular insect In consequence of these facts, the amount of virus present in the mosquito at the end of the incubation period may or may not exceed the initial quantity it had taken up at the time of the infective meal. Whitman and Antunes (1938 *a*) transmitted 2 strains of yellow fever virus by *Aedes aegypti*, the minimal period between the date of the infectious meal and the earliest ability to transmit infection by biting was 14 to 15 days

Effect of temperature on virus multiplication in *Aedes aegypti*.

The influence of temperature in modifying the length of the extrinsic incubation period of yellow fever virus has been carefully studied by Davis (1932). From these researches it emerged that the duration of the interval period could be shortened by the effect of heat, and lengthened by exposing the insects to cold Davis was able to prove that *Aedes* became infective in as short a time as 4 days if they were kept at 37°C, or else in 5 days at 36°C; 6 days at 31°C; 8 days at 25 1°C., or 11 days at 23 4°C., and took as long as 18 days to acquire infectivity when kept at 18°C. He also demonstrated that, if the mosquitoes were maintained at 18°C. for 30 days, they proved to be noninfective to monkeys, but after the same insects had been reincubated for 6 days at room temperature, they then became infective. In another and similar experiment, *Aedes* which had fed on infected blood were stored at 8°C. for 24 days, at the end of which their bites were shown to be noninfective, but after placing them in warmth for 6 days at 36°C, they became infective. The effect of heat above the optimum temperature requisite for the development of infectivity was also studied by Davis, who found that exposure of mosquitoes to 36°C. for 20 days, followed by 40°C. for 7 hours, appeared to attenuate the pathogenicity of the virus (see also Dinger, *et al.*, 1929 *a*)

Effect of temperature on virus multiplication in *Haemagogus*.

Three-days-old mice inoculated subcutaneously were employed for quantitative assay of virus by Bates and Roca-Garciá (1946 *b*, 1945). Following a large meal, at 20°C, the quantity of virus ingested immediately began to decline and continued to do so until it reached a minimum in 5 days, at which level it remained indefinitely. If the meal was small, infection failed to establish itself in the insect. At 25°C. the lowest level was recorded on the 4th day, and at 30°C. in 2 days. After the minimum titer was attained, virus multiplication progressively increased in amount and adequate virus was present to infect a monkey after 10 days at 30°C, 12 days at 25 to 35° C, and 28 days at 25° C. Mosquitoes became infective after 23 days when incubated at alternate and varying temperatures of 25°C. for 20 hours and 30°C. for 4 hours daily. The temperature range 25° to 35°C. approximated most closely the diurnal fluctuation limits of the forest canopy and appeared to prolong the life of the mosquitoes in addition to encouraging egg laying by the female Much individual variation was noted to occur in the susceptibility of different

TABLE 17—*Continued*

RESULTS FROM OTHER PARTS OF THE WORLD

Species	Country of Origin	Results of Biting Test: Positive (+) Negative (o)	Results of Biting Test: Least number of days positive	Results of Inoculation Test[1]: Positive (+) Negative (o)	Results of Inoculation Test[1]: Greatest number of days positive	Authority
Aedes (Stegomyia) aegypti (Linn.)	Cuba	+	12–4	—	—	Reed *et al.* (1911)
Aedes (Stegomyia) aegypti (Linn.)	India	+		—	—	Hindle (1929)
Aedes (Stegomyia) albopictus (Skuse)	Java	+	?	—	—	Dinger *et al.* (1929)
Aedes (Stegomyia) scutellaris (Walker) (syn. *albopictus*)	Far East	+	?	—	—	de Vogel (1930)
Mansonia (Mansonioides) africanus (Theo.)	Africa	+	16	—	—	Philip (1930)
Aedes geniculatus (Oliv.)	France	+	14	—	—	Roubaud *et al.* (1937)

[1] The sign (—) signifies no result recorded

Identification of the blood meal in the mosquito.

Some varieties of mosquito feed only on the blood of a single animal, but others feed on a multiplicity of species The type of blood ingested can be identified by means of serological precipitin tests, and from the results it is possible to tell from what kind of animal the mosquito has derived its meal. Davis and Philip (1931) performed precipitin tests for evidence of human hemagglutinins on a range of West African yellow fever vectors, and concluded that *Taeniorhynchus* (*M*) *africanus* and *Anopheles gambiae* fed exclusively upon man, since their stomach contents gave positive reactions only for human cells Likewise, *A. pharoensis*, *A. funestus*, and *Culex thalassius* were also proved to have fed on human blood *C. nebulosus*, a common type of domestic breeding mosquito, was found to contain only chicken blood, but *C. decens*, *C rima*, *T. annetti*, and *Uranotaenia annulata* reacted with both human and avian sera.

POSSIBLE SPREAD OF YELLOW FEVER BY TICKS

Ticks have also been shown to be capable of transmitting yellow fever under-laboratory conditions, and the results of tests conducted on various species are summarized in Table 18 Perhaps one of the most remarkable observations made on this subject has been the report of Aragão (1933), who demonstrated the presence of yellow fever virus in the eggs of *Amblyomma cajennense*, 11 days after being deposited by a female which had fed upon an infected monkey 25 days previously.

Experimental work by Monteiro (1930) suggests that the bedbug *Cimex lectularius* is able to carry the virus for a short period of time

Certain other species of tick are incapable of transmitting yellow fever virus, and Kumm and Frobisher (1932) found that the bedbug *Cimex hemipterus* killed and destroyed the virus after it had ingested infected blood

The ability of dog fleas to transmit yellow fever virus was studied by Hoskins (1934) who reported that *Ctenocephalides canis* (Curtis) did not transmit the virus to monkeys in interrupted feeding experiments, but fleas which had fed on an infected animal 7 hours previously, when injected into rhesus monkeys proved infective Eighteen hours after an infective blood meal the virus was not demonstrable in the bodies of fleas In an interrupted feeding experiment, biting stable flies of the species *Stomoxys calcitrans* (Linn) were also found to harbor the virus for 6 hours, but were unable to transmit it for 16 hours Flies which had fed on monkeys 42 hours previously, when ground up and injected into monkeys, proved to contain the virus, but 48 to 72 hours later the virus disappeared

TABLE 18

RESULTS OBTAINED WITH TICKS AND TRIATOMA

Species	*Locality*	*Greatest Number of Days Positive*	*Method of Testing*	*Result*	*Authority*
Amblyomma cajennense (Fabricius)	São Salvador Bahia	15	Injection	Pos	Davis (1933)
	Rio de Janeiro	3	Bite	Pos	Aragão (1933)
Argas persicus (Oken)	Oswaldo Cruz Rio de Janeiro	6	Injection	Pos	Davis (1933)
Boophilus microplus (Canestrini) (syn *annulatus*)	São Salvador Bahia	10	Injection	Pos	Davis (1933)
Ornithodorus moubata (Murray)	Rio de Janeiro	4	Bite	Pos	Aragão (1933)
Ornithodorus rostratus (Beaurepaire-Aragão)	Rio de Janeiro	4	Bite	Pos	Aragão (1933)
Rhipicephalus sanguineus (Latreille)	São Salvador Bahia	23	Injection	Pos	Davis (1933)
Triatoma megista (Burm)		7	Injection	Pos	Davis and Shannon (1931)

Haemagogus to infection and length of time required to reach it. Relatively short exposures to high temperatures in nature may greatly hasten virus development. Bates and Roca-Garciá (1945), reported that the extrinsic incubation period of yellow fever virus in *Haemagogus capricornii*, was found to be 22 to 24 days at 24°C. to 27°C., and 13 to 15 days at 30°C. They also mentioned that the habits of the Saimiri monkeys of the Villavicencio area corresponded closely with those of the *Haemagogus* mosquito. See also Bates and Roca-Garciá (1946 *c*).

Contact infection, transovarial passage, feeding with immune sera and other mosquito experiments.

Persistence of yellow fever virus in mosquitoes after death was studied by Wadell (1947), and virus was recovered from *H. equinus* and *H. spegazzinii* from 24 to 72 hours after death. Ether was recommended for killing insects in such tests.

There is no proof that the virus is transmitted from infected to healthy mosquitoes. Aragão (1929) and Kerr and Hayne (1932) state that *occasionally this is possible under laboratory conditions*, but express the opinion that it is *unlikely* that it occurs normally in nature. Hindle (1930) likewise expressed the same opinion and pointed out that, since the male insect did not feed on blood, it probably became infected through surface contact with the feces discharged from infected female mosquitoes, and such superficial contamination could be washed away from the insect's body. The results of Aragão and Costa Lima (1929 *a*, 1929 *b*, 1929 *c*) lend additional support to Hindle's view, since they have demonstrated the existence of *the virus in the dejecta of infected mosquitoes* by inoculating rhesus monkeys with such material. Under experimental conditions it seems possible that the virus can be transmitted by an infected female *Aedes* via eggs to a second generation of mosquitoes. The pioneer investigators, Marchoux and Simond (1906 *a*), suggested that an infected *A. aegypti* could live for 30 days and lay 7 batches of eggs, of which those deposited after the 12 days' extrinsic incubation period became infective 14 days after they had hatched as imagos. It is probable, however, that this does not occur normally in nature, but only under certain laboratory conditions. Davis and Shannon (1930) have investigated the question of the possible spread of yellow fever virus among mosquitoes, and concluded from their studies that it was unable to pass from an infected mosquito through its eggs to a second generation of insects. Further there was no evidence that adult mosquitoes which had been bred from larvae fed on infected mosquitoes could become infective. Whitman and Antunes (1938 *b*) infected half-grown *Aedes aegypti* larvae with a mixture of infected rhesus serum and saline containing 30,000,000 mouse MLD per c.c. Results showed that if unusually large injections such as this were employed the virus of yellow fever could be induced to persist throughout.

Another interesting point investigated in connection with mosquitoes was the experiment conducted by Davis (1931 *a*), who attempted to determine whether *A. aegypti* which had been fed upon the blood of an immune animal would subsequently prove nonreceptive to yellow fever virus contained in infected blood. The tests were negative, for, although the insects were temporarily refractory to infection after being fed on immune blood, after blood became digested *in the* intestinal tract of the mosquitoes, they again acted as carriers of infection.

As mentioned before, personal precautions are necessary for laboratory personnel handling infected mosquitoes, and some of the technical measures adopted for insuring the safety of investigators have been described in a paper by Sellards (1932). This worker has maintained a virulent strain of virus in the laboratory by frequent passage through mosquitoes and monkeys over a period of 3½ years, without accidental *infection*. Laboratory-bred *H. spegazzinii* have been shown to feed readily on brewer's yeast by Bates (1947).

mosquitoes failed to transmit the disease to *Cercopithecus tantalus*, *Cercocebus torquatus*, *Cercopithecus mona*, and *Erythrocebus patas* monkeys, although the blood of these animals gave a negative mouse protection test and contained no yellow fever antibodies. But it was shown that the virus survived in the blood stream of the first 2 named species and these were capable of infecting *A. aegypti*, and, in the case of the 3rd variety, *C. mona*, the blood of the animal proved pathogenic to a rhesus monkey. At the end of the experiment, the sera of all 4 types of monkey were tested again, and now found to have developed neutralizing antibodies for yellow fever virus and were able to confer passive protection on rhesus animals against infection. The existence of inapparent yellow fever infections in monkeys is also referred to by Petit (1929) and Theiler and Hughes (1935). The last-mentioned pair of workers employed the African green monkey of the species *Lasiopyga callitrichus* and observed that if either modified or unmodified strains of yellow fever virus were inoculated intraperitoneally in this variety, the virus multiplied in the blood stream of the animal without giving rise to signs of illness. If, on the other hand, the monkey was injected intracerebrally either with neurotropic or viscerotropic virus, then fatal encephalitis ensued Data are summarized in Table 19.

TABLE 19

SOME EXPERIMENTAL WORK ON DIFFERENT SPECIES OF MONKEY

Species of Monkey[1]	Place of Origin	Number of Animals Investigated	Protection test Result before Experimental Infection	Method of Infection	Results of Infection: Symptoms	Deaths	Liver Lesions	Protection test Result after Experimental Infection	Completion of Mosquito monkey mosquito Cycle	Authority
Alouatta caraya (Humboldt)	Amazon Valley	2		Blood injection	−					Davis (1931 *b*)
Alouatta seniculus (Linn)	Amazon Valley	4	−	Mosquito bite	+		−	+	+	Davis (1931 *b*)
Aotus trivirgatus (Humboldt)	Brazil	1	−	Bite	−	−		+	−	Davis (1931 *b*)
Ateles ater (F Cuvier)	Brazil	5	−	Bite and injection	+	−	−	+	−	Davis (1930 *b*) Davis (1931 *b*)
Ateles paniscus (Linn)	Amazon Valley	1		Injection	+	+	+	•		Lloyd and Penna (1933)
Ateles variegatus (Wagner)	Amazon Valley	1		Injection	+	+	+			Lloyd and Penna (1933)
Cacajao rubicundus (I Geoffroy)	Brazil	3	+	Bite and injection	−	−		+	−	Davis (1931 *b*)
Callicebus moloch (Hoffmannsegg)	Brazil	2		Injection	+		+	+	+	Davis (1931 *b*)
Callithrix albicollis (Spix)	Bahia	26	−	Bite and injection	+	+	−	+	+	Davis (1930 *c*) Lloyd and Penna (1933)
Cebus frontatus (Kuhl)	Brazil	77	±	Bite and injection	+	±	±	+	+	Davis (1930 *a*) Davis and Shannon (1929 *a*)
Cebus variegatus (E Geoffroy)	Brazil	3		Bite and injection	±	−		+	+	Davis (1931 *b*) Lloyd and Penna (1933)
Lagothrix lagotricha (Humboldt)	Brazil	12	−	Injection	+	−	−	+	+	Davis (1930 *b*)
Leontocebus ursulus (Humboldt) = *Cercopithecus ursulus* (Elliot)	Para	10		Bite and injection	+	+	+		+	Davis (1930 *c*) Lloyd and Penna (1933)
Pithecia monachus (E Geoffroy)	Brazil	2		Bite and injection	+	−		−	+	Davis (1931 *b*)
Pseudocebus azarae (Rengger)	Brazil	2		Injection	+	−	−			Aragão (1929 *a*)
Saimiri sciureus (Linn)	Brazil	34	−	Bite and injection	+	+	+	+	+	Davis (1930 *b*) Lloyd and Penna (1933)

[1] See footnote to Table 20, p 498

The *M rhesus* monkey is one of the most uniformly susceptible of all varieties, and Bauer (1931) found that the blood of one infected animal was infective to another even up to a dilution of 1 in 1,000,000,000 parts Lewis (1930) also suc-

ROUTES OF INFECTION WITH YELLOW FEVER

The virus appears to be able to pass through unbroken skin, and Low and Fairley (1931) have described 2 cases affecting laboratory assistants in London, one of whom acquired yellow fever through contact with infected blood, while performing a blood count, and the other when conducting a biochemical analysis.

Aragão and Costa-Lima (1929 *a*, 1929 *b*, 1929 *c*) showed that the feces of infected *Aedes* were capable of transmitting the disease to rhesus monkeys, if the material was rubbed into the skin or placed in contact with the surface of the conjunctiva. During the conduct of a postmortem examination on a fatal case of yellow fever, the risk of nonimmune persons developing infection is considerable, and the use of strong rubber gloves is imperative for personal safety.

Direct contact as a possible avenue of infection has been investigated thoroughly by the United States Army Commission, who studied this question during the course of an epidemic which affected Cuba in 1900. As the result of their inquiries they succeeded in proving the noncontagious character of yellow fever infection, and it was shown that cases of the disease could be tended by nonimmune nurses without risk of infection spreading. The same Commission also eliminated fomites as a possible means of conveying infection. Thus, during the progress of an epidemic which affected the members of a military garrison at Pinar del Rio in Cuba, it was revealed that nonimmune persons who had either slept in the beds vacated by the sick, or washed the soiled clothing of dead patients did not contract the disease.

The inability of fomites to transmit infection was convincingly demonstrated in the classical experiments of Dr Cook and other members of the United States Army Commission, who proved that it was possible for a healthy person to sleep for 20 consecutive nights amid bedclothing consisting of pillows, sheets, and blankets which had been deliberately soiled with the black vomit, urine, and feces of yellow fever cases, without becoming infected In one of these experiments two young, susceptible, American volunteers are reported to have slept for 21 nights wearing garments which had been removed from yellow fever patients, in addition to using their much-soiled pillow slips, sheets, and blankets

PATHOGENICITY TO ANIMALS

Chimpanzee

When working at Mañaos, Thomas (1907) reported that he was able to infect a chimpanzee with yellow fever virus Twenty-nine infected specimens of *Aedes aegypti* were permitted to bite the ape, and after an incubation period of 21 days the animal developed a febrile illness which closely resembled that of the human disease

Monkeys

Since Stokes, Bauer, and Hudson (1928) and Mathis, Sellards, and Laigret (1928) discovered the susceptibility of *Macacus rhesus* to experimental infection, other Indian monkeys such as *M sinicus*, *M cynomolgus*, and *M nemestrinus* were likewise found to succumb to inoculation African monkeys, on the other hand, appeared to prove resistant to the virus, and for a time it was thought that such animals owed their freedom from attack to a natural racial immunity. It is now appreciated that this is not true, for many of the African species of animal do not possess a natural immunity, but acquire it at some period of their life in the forest (see Findlay, Stefanopoulo, Davey, and Mahaffy, 1936)

Different species of monkey vary in their susceptibility to infection independent of the presence of serum protective antibodies For example, Bauer and Mahaffy (1930) found that the inoculation of infective rhesus blood and bites of infected

mosquitoes failed to transmit the disease to *Cercopithecus tantalus*, *Cercocebus torquatus*, *Cercopithecus mona*, and *Erythrocebus patas* monkeys, although the blood of these animals gave a negative mouse protection test and contained no yellow fever antibodies But it was shown that the virus survived in the blood stream of the first 2 named species and these were capable of infecting *A. aegypti*, and, in the case of the 3rd variety, *C. mona*, the blood of the animal proved pathogenic to a rhesus monkey. At the end of the experiment, the sera of all 4 types of monkey were tested again, and now found to have developed neutralizing antibodies for yellow fever virus and were able to confer passive protection on rhesus animals against infection. The existence of inapparent yellow fever infections in monkeys is also referred to by Pettit (1929) and Theiler and Hughes (1935). The last-mentioned pair of workers employed the African green monkey of the species *Lasiopyga callitrichus* and observed that if either modified or unmodified strains of yellow fever virus were inoculated intraperitoneally in this variety, the virus multiplied in the blood stream of the animal without giving rise to signs of illness. If, on the other hand, the monkey was injected intracerebrally either with neurotropic or viscerotropic virus, then fatal encephalitis ensued Data are summarized in Table 19

TABLE 19

SOME EXPERIMENTAL WORK ON DIFFERENT SPECIES OF MONKEY

Species of Monkey[1]	Place of Origin	Number of Animals Investigated	Protection test Result before Experimental Infection	Method of Infection	Results of Infection: Symptoms	Deaths	Liver Lesions	Protection test Result after Experimental Infection	Completion of Mosquito monkey-mosquito Cycle	Authority
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Alouatta seniculus (Linn.)	Amazon Valley	4	−	Mosquito bite	+		−	+	+	Davis (1931 *b*)
Aotus trivirgatus (Humboldt)	Brazil	1	−	Bite	−	−		+	−	Davis (1931 *b*)
Ateleus ater (F Cuvier)	Brazil	5	−	Bite and injection	+	−	−	+	−	Davis (1930 *b*) Davis (1931 *b*)
Ateleus paniscus (Linn.)	Amazon Valley	1		Injection	+	+	+	•		Lloyd and Penna (1933)
Ateleus variegatus (Wagner)	Amazon Valley	1		Injection	+	+	+			Lloyd and Penna (1933)
Cacajao rubicundus (I Geoffroy)	Brazil	3	+	Bite and injection	−	−		+	−	Davis (1931 *b*)
Callicebus moloch (Hoffmannsegg)	Brazil	2		Injection	+		+	+	+	Davis (1931 *b*)
Callithrix albicollis (Spix)	Bahia	26	−	Bite and injection	+	+	−	+	+	Davis (1930 *c*) Lloyd and Penna (1933)
Cebus frontatus (Kuhl)	Brazil	77	±	Bite and injection	+	±	±	+	+	Davis (1930 *a*) Davis and Shannon (1929 *a*)
Cebus variegatus (E Geoffroy)	Brazil	2		Bite and injection	±	−		+	+	Davis (1931 *b*) Lloyd and Penna (1933)
Lagothrix lagotricha (Humboldt)	Brazil	12	−	Injection	+	−	−	+	+	Davis (1930 *b*)
Leontocebus ursulus (Humboldt) = *Cercopithecus ursulus* (Elliot)	Para	10		Bite and injection	+	+	+		+	Davis (1930 *c*) Lloyd and Penna (1933)
Pithecia monachus (E Geoffroy)	Brazil	2		Bite and injection	+	−		−	+	Davis (1931 *b*)
Pseudocebus azarae (Rengger)	Brazil	2		Injection	+	−	−			Aragão (1929 *a*)
Saimiri sciureus (Linn.)	Brazil	34	−	Bite and injection	+	+	+	+	+	Davis (1930 *b*) Lloyd and Penna (1933)

[1] See footnote to Table 20, p 498

The *M rhesus* monkey is one of the most uniformly susceptible of all varieties, and Bauer (1931) found that the blood of one infected animal was infective to another even up to a dilution of 1 in 1,000,000,000 parts. Lewis (1930) also suc-

TABLE 20

IMMUNITY TESTS ON WILD MONKEYS

Species[1]	Place of origin	No tested	Result Neg	Result Pos	Authority
Allenopithecus nigroviridis Pocock Dwarf green monkey	Belgian Congo	1	1	—	Findlay *et al* (1936)
Anthropopithecus troglodytes Linn chimpanzee	French Guinea	6	5	1	Findlay *et al* (1936)
Callicebus ornatus	Restrepo, Colombia	4	1	3	Soper (1936)
Cebus apella	F Bananal, Patos	57	49	8	Soper (1936)
Cebus apella	Deputado	1	—	1	Soper (1936)
Cebus apella	Turriel	3	—	3	Soper (1936)
Cebus apella	F Rio Verde, Campina Verde	1	1	—	Soper (1936)
Cebus apella	Capellinha Chumbo, Patos	1	1	—	Soper (1936)
Cebus apella	F Alberto, Patos	1	1	—	Soper (1936)
Cebus apella	F Patrona, Araguary	1	—	1	Soper (1936)
Cebus apella	Planalto, Matto Grosso Ponte Alta	1	—	1	Soper (1936)
Cebus apella	F Pantano, Patos	20	20	0	Soper (1936)
Colobus badius (sub sp nov)	Goaso, Gold Coast	1	—	1	Findlay *et al* (1936)
Colobus vellerosus (Elliot) (*polykomos* Schwarz)	Gold Coast	5	2	3	Findlay and Mac Callum (1937)
Colobus vellerosus (Elliot) (*polykomos* Schwarz)	Sierra Leone	1	0	1	Findlay and Mac Callum (1937)
Erythrocebus patas (Elliot)	Gold Coast	1	1	0	Findlay and Mac-Callum (1937)
Erythrocebus patas (Schreber) Patas monkey	Mampong, Gold Coast	1	1	—	Findlay *et al* (1936)
Erythrocebus patas (Elliot)	Anglo Egyptian Sudan	1	1	0	Findlay and Mac-Callum (1937)
Galago senegalensis senegalensis (Elliot)	Anglo Egyptian Sudan	3	3	0	Findlay and Mac Callum (1937)
Hapale sp	Minas Geraes, Vendinha, Araguary	2	2	0	Soper (1936)
Mandrillus sphinx (Linn) Mandrill	Gambia	1	1	—	Findlay *et al* (1936)
Papio sp Baboon	Belgian Congo	1	—	1	Findlay *et al* (1936)
Procolobus badius badius (Pocock, 1935)	Gold Coast	6	3	3	Findlay and Mac-Callum (1937)
Procolobus badius waldroni, (Hayman)	Gold Coast	3	2	1	Findlay and Mac-Callum (1937)
Saimiri sciureus (Linn)	Restrepo, Colombia	1	—	1	Soper (1936)
Cercopithecus aethiops centralis (Neumann)	Uganda	20	15	5	Findlay and Mac-Callum (1937)
Cercopithecus aethiops centralis (Neumann)	Anglo-Egyptian Sudan	15	14	1	Findlay and Mac Callum (1938)
C aethiops cynosuros (Scopoli) Malbrouck guenon	Belgian Congo	1	1	—	Findlay *et al* (1936)
C aethiops sabaeus (Linn) Green monkey	Bathurst, Gambia	4	4	—	Findlay *et al* (1936)
C cephus (Linn) Moustached guenon	Belgian Congo	2	2	—	Findlay *et al* (1936)
Cercopithecus diana roloway (Schreber) Gold Coast Diana monkey	Goaso, Gold Coast	1	1	—	Findlay *et al* (1936)
Cercopithecus diana diana	Gold Coast	1	0	1	Findlay and Mac-Callum (1937)
C galeritus agilis (E Riviere) Agile mangabey	Belgian Congo	4	4	—	Findlay *et al* (1936)
C mona lowei, *Thomas mona monkey*	*Goaso, Gold Coast*	1	1	—	Findlay *et al* (1936)
C neglectus (Schlegel), Schlegel's guenon	Belgian Congo	1	1	—	Findlay *et al* (1936)
Cercopithecus sp	Gold Coast	1	1	0	Findlay and Mac-Callum (1937)
Cercopithecus sp	Liberia	10	6	4	Findlay and Mac Callum (1937)

[1] The nomenclature employed in Tables 19 and 20 is not always in accordance with the latest scientific usage, but to avoid dubiety we have adhered to the names employed by the original authors We have also observed that a considerable variation in spelling has been indulged in by different workers describing a single species of monkey sometimes even in the same paper

ceeded in infecting monkeys with as little as 0 000001 c c of infected blood Infection may be produced with human or mouse neurotropic yellow fever virus given by any route of parenteral injection, as well as through the bites of infected mosquitoes, and Findlay and Clarke (1935) showed that, if the virus was instilled into the nose, it could be recovered from the olfactory lobes two days later, after which it became distributed throughout the brain Rhesus monkeys have also been infected with the excreta of infected Aedes mosquitoes by inoculating the material subcutaneously or else by placing it on the surface of the conjunctiva Aragão and Costa Lima (1929 *a*, 1929 *b*, 1929 *c*) have thus drawn attention to the ability of the virus to pass through intact skin and mucous membrane surfaces The incubation

period depends on the dose of virus given and may be as short as 3 or as long as 31 days (Frobisher, 1931 *c*).

If a strain of neurotropic virus be injected subcutaneously or intraperitoneally,

passages were effected, with considerable modification of the properties of the virus (Penna, 1936)

Certain South American species such as *Saimiri sciureus*, *ateleus*, *Cebus frontatus*, *callithrix*, and *Leontocebus ursulus* have also proved susceptible, when inoculated intracerebrally with Berkefeld filtrates of infective tissue (Lloyd and Penna, 1933, and Lloyd and Mahaffy, 1936)

The presence of antibodies in the sera of a proportion of monkeys recovered from an infected area has been studied and Table 20 contains a summary of results

The disease in rhesus monkeys is accompanied by fever, which varies from 104° to 105° F, lasts for 2 days, and frequently results in death During this illness hemorrhagic symptoms similar to those evident in man, such as bleeding from the gums, the characteristic black vomit, and icterus visible in the conjunctivae, are present The urine contains granular and hyaline casts accompanied by albumin and bile The naked-eye and microscopic lesions occurring in experimental yellow fever in the rhesus monkey have been described and compared with the changes produced in man by Hudson (1928, 1928 *a*, 1928 *b*)

With minor differences, the *pathological changes* found in the monkey resemble

are similar to the human appearances, but polymorphonuclear leukocytes and endothelial cells are more numerous In the kidney, congestion of the glomerular vessels and the presence of granular, calcareous, and hyaline casts are less common than in man (Hindle, 1930 *a*) Lesions present in the heart are similar to those found in man and have been described on p. 470

Goodpasture (1932) studied the morbid histology of yellow fever encephalitis in rhesus monkeys after intracerebral injection of virus, and reported that the essential lesion was acute disseminated encephalomyelitis, which extended throughout the brain and caused necrosis of both motor and sensory ganglion cells Intranuclear inclusion bodies were also detected in these cells Findlay and Stern (1935) made similar observations and found that the lesions in monkeys resembled those described by them in mice (see p 500)

The cerebrospinal fluid in infected monkeys has been examined by Mollaret and Stefanopoulo (1934), and, in a few of the animals which had been inoculated subcutaneously with neurotropic virus, the yellow fever virus was recovered from the fluid Eighteen days after inoculation the lymphocyte count rose to 67 2 per c mm and the Pandy test was positive

The regeneration of damaged liver and kidney tissue following recovery from experimentally induced yellow fever has been studied in 6 rhesus monkeys by Klotz and Belt (1930 *b*) On every occasion it was found that the tissue had undergone complete repair without any indication of cirrhosis in the liver, or scarring or contraction of the kidney According to these workers, the absence of fibrosis in the liver and kidney has been ascribed to the peculiar resistance of stroma tissue against injury with yellow fever virus The pathological process is essentially one which is noninflammatory and nonautolytic in character, and which neither gives rise to the formation of thrombi in the small vessels of the parenchyma, nor provokes a connective tissue reaction during the acute phase of the illness The process of regen-

eration takes place quickly and originates from the surviving islands of parenchymal tissue, and from the results obtained in monkeys, Klotz and Belt have concluded that recovery in man likewise pursues the same course.

Chemical and metabolic changes in the infected rhesus monkey have been studied by Wakeman and Morrell (1931). These observers found that the sugar tolerance of the animal was reduced prior to death, and dextrose injected into the blood of monkeys suffering from yellow fever was not as readily removed by the blood stream as was the case when similar amounts were introduced into normal monkeys. Wakeman and Morrell thus demonstrated the loss of hepatic function during yellow fever infection in monkeys The icteric index in rhesus monkeys has been estimated by Kerr (1931), who found that the degree of icterus present on the 2nd and 3rd days of the animal's illness was of diagnostic and prognostic significance For example, if the reading was 10 or more units on the 2nd day, and 15 or over on the 3rd or 4th day of the animal's illness, it usually indicated a fatal termination. The bromsulfalein test was also performed by Kerr (1931), who found the retention of 10 per cent. or more of the dye on the 2nd day of disease, and 15 or greater per cent. on the 3rd day, proved to be a fatal prognostic sign In com- [illegible] to determine [illegible] phenomena [illegible] ttack of the disease derive a lifelong immunity to reinfection. Passive immunity may also be conferred in monkeys by injection of immune serum and Bauer (1931 *a*) points out that this is of long duration if homologous immune serum is employed

Opossums (South American)

Following infection, *Didelphis marsupialis* (common opossum); *D. paraguayensis* (white-eared common opossum) and *Metachirops opossum* (gray-masked opossum) occasionally showed circulating virus in peripheral blood. *M. nudicaudatus* (brown-masked opossum) *Caluromys philander* (woolly opossum), *M cinera*, and *M. incana* (murine opossum) are susceptible (Laemmert, 1946).

Mice

The virus is pathogenic to these animals when injected intracerebrally According to the investigations of Findlay and Stern (1935), neurotropic yellow fever virus, when injected intracerebrally in mice, causes an inflammatory reaction of the central nervous system accompanied by degeneration of nerve cells, the formation of acidophilic intranuclear inclusion bodies, infiltration with mononuclear cells, and proliferation of microglia. Varying stages of degenerative changes were evident in the ganglion cells, such as swelling of the cells, neuronophagia or complete disintegration of their contents Inclusion bodies were seen only in nerve cells which had been extensively damaged, and they failed to stain by the Feulgen technique No evidence of demyelination was observed at any stage of the pathological process (see also Theiler, 1930, and Mathis, 1936) The virus has also been passaged intratesticularly by Lloyd and Mahaffy (1933), and later Smith (1938) carried out 40 passages in this manner When administered by this route it causes slight swelling of the organ, accompanied by an increase in the amount of virus up to the 7th day, after which it declines and disappears completely on the 21st day Smith (1938) reported that yellow fever virus may thus be isolated from infected serum by inoculating mice either intracerebrally or intratesticularly Laigret (1933) employed different strains of mice, namely *Mus musculus*, *M gentilis*, and *M musculus azoricus*, and was able to produce encephalomyelitis in these animals by inoculating them intraperitoneally with neurotropic virus (see also Nicolau, Kopciowska, and Mathis, 1934 *a*, Stefanopoulo, 1934, and Mathis, 1936 *a*) The paths of spread of yellow fever virus in mice have been studied by Findlay and Mahaffy (1936) From their researches they have concluded that the virus, when administered intra-

peritoneally, does not reach the central nervous system centripetally *via* the spinal nerves, but does so via the peripheral circulation, through the nasal mucosa and the olfactory nerves The latter observation is supported by the fact that, when yellow fever virus is introduced intraperitoneally into very young mice, the intranasal instillation of picric acid arrests the spread of infection to the central nervous system Mice have also been infected by instilling neurotropic yellow fever virus into the conjunctival sac of the eye (Findlay and Clarke, 1935).

Guinea-pigs

Theiler (1933) showed that the guinea-pig was susceptible to intracerebral inoculation, and he passaged a strain of neurotropic mouse virus 42 times in this

with a strain of monkey virus were unsuccessful. Immunization of guinea-pigs against neurotropic and 17 D virus by cutaneous scarification has been reported by Favarel (1945).

Hedgehogs

The Sudanese hedgehog (*Atelerix albiventris*) has been successfully infected with viscerotropic yellow fever virus by Findlay, Hewer, and Clarke (1935). Like-

intracerebrally (Smith, 1936) Findlay and Mahaffy (1936), on the other hand, report that the hedgehog *A. albiventris* obtained from Kano in Northern Nigeria was found to be highly resistant to inoculation with neurotropic virus, furthermore, this immunity was not acquired as a result of previous infection, as no antibodies were demonstrable in the blood of one animal examined for this purpose. Two of the animals showed neutralizing properties in their blood after inoculation, and Findlay and Mahaffy concluded that the immunity of the hedgehogs studied by them was a natural and racial characteristic.

The principal pathological changes were seen on microscopic examination of the liver tissue There was fatty degeneration, pigmentation of the Kupffer cells, and diffuse infiltration of the organ with lymphocytes, particularly beneath Glisson's

Bats

The susceptibility of the species *Epomophorous wahlbergi haldemani* (Hallowell) to experimental infection with neurotropic yellow fever virus has been reported by Rodhain (1936) Animals inoculated intracerebrally developed signs of encephalitis in 9 to 10 days, and the virus was thereafter demonstrable in the peripheral blood

Shrews

The Southern Nigerian species (*Crocidura manni*) is apparently resistant to experimental infection with yellow fever virus Smith (1936) showed that although these rodents possessed no protective antibodies in their sera, they nevertheless

could not be infected with a neurotropic strain of virus administered intracerebrally or subcutaneously.

Pigs

Stefanopoulo, Mollaret, and Desnos (1934) showed that the pig was susceptible to intracerebral inoculation with yellow fever virus. Following infection, the animal first showed a rise in temperature reaching 42° C, and later signs of encephalomyelitis, and death in 7 days. Histological sections prepared from the brain of such an animal showed areas of perivascular lymphocytic infiltration or cuffing similar to that caused by other neurotropic virus infections.

Horses

Pettit, Stefanopoulo, and Frasey (1928) found that this animal was resistant to inoculations of yellow fever virus, and after repeated injections the serum of the animal was found to develop antibodies that were able to neutralize yellow fever virus in *in vitro* tests

Young Chickens

These were shown to be susceptible to intracerebral inoculation with the *Asibi* strain of virus which had been maintained in chick embryo tissue for several transfers. One hundred and two direct brain-to-brain transfers were made in chickens Virus appeared in the blood stream on the 6th day and remained in the brain for 14 days. Young chickens never showed signs of encephalitis (Laemmert and Moussatché, 1943).

Birds

In Brazil, Laemmert, de Castro-Ferreira, and Taylor (1946) shot 141 birds representing 45 species and examined the livers for evidence of yellow fever virus but with negative results throughout

Poikilothermal Animals

The following creatures captured in the vicinity of Villavicencio, Colombia, could not be infected with yellow fever virus by Laemmert (1943). The iguanas, *Iguana iguana* (L.), the lizard, *Tupinambis nigropunctatus* (Spix) tortoises, *Testudo tabulata*, Walbium, boas, *Constrictor c constrictor* (L), toads, *Bufo marinus* (L); and tree frogs, *Hyla sp* Likewise, negative were lizards, *Tupinambis teguixin* (L) and frogs, *Leptodactylus ocellatus* (L) gathered from near Rio de Janeiro

Other Animals

The cat, rabbit, rat, ferret, golden hamster (*Cricetus auratus*), wood vole (*Apodemus sylvaticus*), bank vole (*Evotomys glareolus*), pigeon, hen, and canary are resistant to intracerebral inoculation with a strain of neurotropic yellow fever virus There is no evidence, however, that the virus can multiply in these animals and no histological changes have been demonstrated in their brains (Findlay, 1934 *a*).

The red squirrel (*Sciurus vulgaris*) and the field vole (*Microtus agrestis*) are susceptible to intracerebral inoculation with neurotropic virus Findlay (1934 *a*) observed that in the field vole, infiltration of the meninges, perivascular cuffing, and degeneration of ganglion cells were present, but to a lesser extent than in mice

CHAPTER XLIII

IMMUNITY MECHANISMS IN YELLOW FEVER

PROPHYLACTIC IMMUNIZATION

THE EMPLOYMENT of killed virus has given disappointing results in animal experiments and consequently it has been rejected for human inoculation Attention has therefore turned toward devising ways and means of cultivating yellow fever virus such as, for example, by repeated intracerebral passage through mice or prolonged growth in tissue culture, with the object of inducing the agent to acquire fixed affinities for particular types of tissue Thus, a strain of modified virus was derived which was sufficiently attenuated in virulence to render it safe for human administration

Since living virus is essential for production of high-grade immunity it is probable that, before antibodies can develop in the serum of a nonimmune subject, the individual must first pass through a subclinical phase of the infection. With the use of live vaccines there is an ever present risk of the virus reverting to its pathogenic properties, but fortunately the occurrence of such incidents has been rare.

Prophylactic vaccination has been evolved from studies originally directed towards insuring the personal safety of investigators engaged in yellow fever research Thus Sawyer, Kitchen, and Lloyd (1932) inoculated a number of workers with fixed mouse virus along with immune human serum, and the efficacy of the procedure was immediately apparent as it terminated the long series of laboratory infections which hitherto had been experienced (see Berry and Kitchen, 1931). Findlay (1934) employed this same method of immunization for 200 cases, and as far as can be determined there are no grounds to suspect that such vaccination has failed to protect persons against an attack of the disease (see also Laigret, Saleun, and Ceccaldi, 1937, Sellards, 1937, Findlay, 1937, and Sellards and Laigret, 1936) From the practical standpoint this method was unsuitable for human inoculation on a large scale, since it necessitated the use of large amounts of immune human serum and, moreover, it was unsafe to administer the virus alone, owing to the possible danger of reversion to its pantropic characteristics For further information on the subject of yellow fever immunization the reader is referred to the writings of Findlay (193[illegible]), Findlay and MacCallum (1937 c), Haagen (1937), [illegible] (193[illegible])

Theiler (1937) showed that the continued propagation of yellow fever virus in tissue cultures led to great modification in invasive properties, so that ultimately it became safe for human immunization See Lloyd, Theiler, and Ricci (1936) and Theiler and Smith (1937)

Cultivation of 17D Strain

The rhesus monkey adapted *Asibi* strain of virus (Lloyd, Theiler, and Ricci, 1936) was passaged by Theiler and Smith (1937) through 18 subcultures in minced chick embryo tissue containing 10 per cent normal monkey serum in Tyrode's solution After the 18th subcultivation, virus was grown for 58 passages in a medium of whole chicken embryo followed by 160 further transfers in medium composed of decerebrated and demyelinated embryos. The resulting virus designated 17D was found to be much reduced in neurotropism and viscerotropism and to be capable

of protecting monkeys against virulent virus. It was also safe enough for human prophylactic inoculation and in November, 1936, Theiler and Smith (1937) inoculated 8 human volunteers with modified 17D virus without the addition of immune human serum, 17D virus could not be transmitted by *Aedes* and the quantity of circulating virus in vaccinated persons was too small to be transmitted by *Aedes* (Whitman, 1939).

The growth characteristics of 17D strain yellow fever virus in cultures of embryonic chick cells in Tyrode's solution has been studied by Fox (1947). The concentration of virus in tissue was found to be 100 times that present in supernatant. The maximum phase of virus multiplication coincided with the greatest period of tissue activity in the culture, namely the first 3 days. By progressively lowering the temperature of incubation from 39.5° C. to 27° C. the peak was delayed until the 8th day. The effects of reduced oxygen tension and size of inoculum also affected virus multiplication Cultures could be prepared and stored in advance at 5° C for 51 days before inoculation without impairment of efficiency. Fox and Laemmert (1947) were also able to cultivate 17D strain virus on 7 to 8 days old embryos by inoculation of dropped chorio-allantoic membrane. Virus first proliferated at the site of infection later invaded the blood stream and thereafter became localized and multiplied in the embryo, particularly in muscle and brain. The presence of sulfapyridine and sulfathiazole in tissue cultures did not interfere with the multiplication of 17D high substrain yellow fever virus (Koprowski and Lennette, 1944 *a*).

Preparation and Assay of 17D Vaccine

According to Smith, Penna, and Paoliello (1938) virus previously cultivated 205 to 287 times in embryos was inoculated in 7-day-old chicken embryos, into the embryo itself, incubated at 37° C. for 3 days and the embryos harvested. Pooled embryos were thereafter triturated in a mortar with abrasive; a 10 per cent. suspension prepared with normal undiluted or 1.50 diluted human serum (now omitted, see Sawyer *et al*, 1944), clarified with coarse filtration, later through a Seitz EK filter; put up in ampules, frozen in alcohol and CO_2 snow, desiccated *in vacuo;* tested for sterility, and stored at 4° C. until required for use. Biological assay of potency and freedom from excessive neurotropism and viscerotropism are determined by intracerebral inoculation of a monkey with 1 c c. Also serial dilutions are inoculated intracerebrally in mice from which the 50 per cent end point activity titer of virus is calculated. The influence of prolonged propagation of the Asibi strain of yellow fever virus in minced chick embryo Tyrode medium was investigated by Bugher and Smith (1944) No difference could be detected in the antigenicity of vaccine lots prepared from material ranging from 212 to 450 serial egg passages Fifty-seven subcultures of 17D strain virus in the presence of specific yellow fever antibody failed to modify its antigenicity in man, as measured by the intraperitoneal mouse protection test. Virus dosage of 10 MLD (50 per cent mortality probability in mice) gave an irregular immunization response in man One hundred LD50 dose was found to be the safe minimum for human inoculation According to Fox, Kossobudzki, and Fonseca da Cunha (1943) the routine vaccinating dose should contain not less than 500 MLD (LD_{50}) for mice (See also de Paula Souza, 1945.)

Storage.

According to Barruss and Hargett (1947), desiccated 17D yellow fever vaccine retains its potency for 3 years at −9° C to −32° C, and 2 years at −5° C to −25° C At 37° C., some 90 to 99 per cent virus activity was lost in 2 to 8 weeks Diluted vaccine ready for inoculation should be shielded from excessive light and heat and be used 1 hour after preparation Should it be necessary to employ vaccine of questionable potency then 10 to 20 times the usual dose should be administered.

Route of inoculation.

The antigenic response in man is most active following intradermal inoculation, and less so by the intramuscular and subcutaneous routes. Provided that an adequate volume is administered, subcutaneous inoculation suffices for practical purposes.

Efficacy of 17D vaccine.

The value of this safe method of immunization in man is now universally recognized. Its introduction among members of the staff of the International Health Division of the Rockefeller Foundation and Special Study sections in Colombia have abolished laboratory infections among workers exposed to intimate contact with virus in the laboratory and field. In the field, Bugher and Gast-Galvis (1944) reported only 1 case of yellow fever among 600,000 persons vaccinated with 17D virus whereas in Colombia for the same period 345 proved and 254 probable infections occurred among unvaccinated persons.

Duration of human immunity following 17D vaccination.

This has been stated to be at least 5 years and probably much longer (Anderson and Gast-Galvis, 1947). Antibodies appear 7 to 10 days after inoculation and last at least 3 years according to Smithburn and Mahaffey (1945). According to Fox and Cabral (1943), among a group of adults inoculated with 17D vaccine 4 years previously, only 2 per cent. failed to show protective response. Similar tests among young individuals showed that after a lapse of 2 and 3 years, nearly 10 per cent. failed to exhibit immunity and a well-marked correlation existed between age and antibody level, the lowest being returned in the case of children under 10 years of age.

Failure of 17D vaccine to protect.

It has been observed that sometimes certain individuals fail to respond to such immunization. The prophylactic value of 17D vaccine was put to a severe test from 1939 to 1945 when thousands of inoculated British and other troops were exposed to infection in the West and other parts of Africa. Elliott (1944) has recorded 3 histologically proved, fatal cases of yellow fever affecting military personnel in West Africa who had been inoculated 2 years previously.

Post Yellow Fever Vaccine Jaundice

Findlay and MacCallum (1937 *a*) first reported that 2 to 3 per cent. of 2,200 persons receiving yellow fever vaccine developed jaundice 2 to 7 months later. They stated that jaundice was not due to infection with yellow fever virus but some other cause, an observation which has since been confirmed, and jaundice is now known to be caused by contamination with the virus of human serum jaundice.

In 1939, jaundice appeared in some 27 per cent. of 304 persons who had been vaccinated with Lot 467 of 17D tissue culture yellow fever vaccine which contained normal human serum in the fluid component of the tissue cultures in which the source of virus was propagated. A further group of 1072 cases of jaundice developed in 1940–41 among 19,000 persons vaccinated in S.E. Espirito Santo. In the light of present knowledge it is certain that the portal of entry of the icterogenic agent was human serum and that Seitz filtration with heating to 56° C. for 30 minutes was inadequate to inactivate the virus of serum jaundice. Later batches of vaccine were prepared without the addition of human serum and no further cases have since occurred (Fox, Manso, Penna, and Madureira-Pará, 1942).

An extensive account of post yellow fever jaundice is given by Sawyer, Meyer, Eaton, Bauer, Putnam, and Schwentker (1944). The incubation period was found to be 60 to 150 days and the highest mortality 3 per 1,000 cases. These workers recommended that serum-free yellow fever virus should be used for immunization

Encephalitis after Yellow Fever Vaccination

Signs of central nervous system involvement following yellow fever immunization have been reported frequently in the literature. Fortunately, the percentage of individuals who develop these unpleasant reactions is very small, and the prospect of encountering such symptoms after inoculation should not be accepted as a valid excuse for refusing prophylactic immunization. For an account of various nervous reactions following anti-yellow-fever vaccination the reader is referred to the work of Barraux, Montel, and Bordeo (1936), Laigret (1936), Darre and Mollaret (1936), Mollaret and Findlay (1936), Lhermitte and Freibourg-Blanc (1936), and Peyre and Fricaud (1937).

Although reports from various quarters have indicated that mouse brain virus can be used with safety for human immunization against yellow fever, the recent work of Findlay and MacCallum (1938) has revealed that even though it may be possible to obtain a neurotropic strain of yellow fever virus for mice, such neurotropism may be confined to the mouse alone and not apply, for example, to the monkey. Thus, it was shown by these workers that even after a strain of virus had been passaged 670 times through the brains of mice, when inoculated into monkeys it produced death with viscerotropic lesions. The liability of yellow fever virus to undergo spontaneous change in its affinities for different tissues is, therefore, a property which must be remembered, and Findlay and MacCallum accordingly have advocated that all strains of virus employed for human immunization should be tested periodically for pathogenicity to insure that no alteration in characters has occurred.

Fox, Lennette, Manso, and Souza-Aguiar (1943) reported that a single substrain of 17D virus was observed to undergo "variation" with the result that of 55,073 persons vaccinated in the arm, 199 of these (0.50 per cent.) presented evidence of central nervous system involvement, with 1 fatality

Allergy to Egg Protein

Two severe cases have been reported by Sprague and Barnard (1945) but mild reactions are not infrequent Individuals susceptible to asthma, urticaria and other allergic phenomena are likely to develop massive edema, dyspnea and dysphagia following parenteral inoculation of egg protein

Use of live neurotropic mouse brain adapted virus (Dakar vaccine).

Sawyer (1937) and Sawyer, Kitchen, and Lloyd (1932) employed mouse brain adapted yellow fever virus combined with immune human or monkey serum Owing to the inconvenience of obtaining adequate amounts of the latter for large scale vaccine production, the method was discontinued Sellards and Laigret (1936) vaccinated humans with similar virus devoid of immune serum but because of undesirable neurotropic effects and adverse reports by Findlay (1934 *b*), Theiler and Whitman (1935 *b*) doubt was cast on the advisability of its continuation The experiences of French workers are summarized by Mathis (1938) and Stefanopoulo (1937). At the time of writing, the latest method of preparation and inoculation practiced at the Pasteur Institute, Dakar, consists of the application of neurotropic mouse brain virus by scarification and in a suspension of gum arabic

Comparative value of Dakar mouse brain and 17D chick embryo yellow fever vaccine.

Tests were conducted under the auspices of UNRRA with the following findings Neurotropic mouse brain live yellow fever virus supplied by the Pasteur Institute, Dakar was tested and found to have an intracerebral mouse infection potency of 10,422 LD50 per c c Virus was suspended in gum arabic, frequently combined with vaccinia virus and administered by scarification to 2 groups of 200

French soldiers each. Group A received neurotropic mouse brain vaccine in gum arabic by scarification, Group B received similar virus to which was added anti-smallpox vaccinia virus vaccine

A 3rd group of men was inoculated with 17D egg embryo virus, the infective titer of which was 49,506 LD50 per c c. Results of immunity tests showed that of Group A, 96.88 per cent. developed neutralizing antibodies, Group B showed 97 47 per cent. and Group C, who received 17D virus, showed 55 60 per cent. positive sera by the intracerebral mouse protection test, and 97–100 per cent. by the intraperitoneal test. de Paula Souza (1946), therefore, concluded that "under the conditions of the experiment the highly neurotropic Dakar vaccine, when inoculated by scarification of the skin, gave a higher degree of immunity, as expressed in the amount of protective antibody produced in their blood, than did less neurotropic 17D virus when injected subcutaneously. There were thus two important variables and the experiment as set up could not show the separate effects of the type of virus and the method of inoculation"

It is interesting to note that although 17D egg embryo virus contained almost 5 times as many LD50 doses as Dakar vaccine in man, the immunological response of the former was much less than the latter Over 14,500,000 persons have been inoculated in the French colonies with the Dakar vaccine.

For obvious reasons 17D has much to commend its use since it is less liable to become contaminated with extraneous live viruses which may gain access to mouse brain tissue

Egg Yolk and Olive Oil Treated Vaccines

In the past, virus coated with a film of olive oil and egg yolk has been tried, and a single dose equivalent to 320 M I D for mice administered. Nicolle and Laigret (1935) inoculated 10,000 persons by this method, and recorded only 2 non-fatal cases of meningitis with 1 of myelitis (see also Nicolle and Laigret, 1936, and Laigret and Durand, 1936) A modification of the former employing egg yolk was described by Mathis, Durieux, and Mathis (1936)

Experiences Reported with Killed Virus

Findlay (1934) found that virus inactivated by the photodynamic action of
and Hughes (1936)
killed by exposure
the administration
l to the course of
injections.

ANIMAL PROTECTION TESTS

The development of antibodies in the serum of a human subject or a monkey after an attack of yellow fever has long been recognized (Stokes, Bauer, and Hudson, 1928) and convalescent serum has been shown to exert powerful *in vivo* and *in vitro* neutralizing effects upon yellow fever virus Stokes and his colleagues found that even as little as 0 1 c c of convalescent human serum was sufficient to protect a monkey against a simultaneous dose of live virus This important discovery has been utilized by later workers to form the basis of biological tests for the detection of neutralizing antibodies in human, monkey, and other varieties of mammalian sera Monkey protection tests were, however, unsatisfactory for the reason that the cost of the animals prohibited their employment in extensive field investigations, and it was not until 1930 that the researches of Theiler (1930) led the way to a further possibility in the study of protection tests Theiler showed that yellow fever virus, provided it was inoculated intracerebrally, was pathogenic to the white mouse. He proved further that the repeated intracerebral passage of the virus in mice led to its adaptation for the central nervous system, so that ulti-

mately a strain of virus was derived exhibiting a fixed neurotropism for mice, following its being administered by any route.

The Intracerebral Mouse Protection Test

Using neurotropic mouse virus, Theiler (1930) showed that the existence of protective antibodies in human sera could be demonstrated by inoculating a mouse intracerebrally with a mixture consisting of virus and antiserum. Notwithstanding the effect of immune serum, and the smallness of the dose of virus (a 1 per cent. suspension of brain), the test was not delicate enough as there was a tendency for the mice to succumb at too irregular intervals. When using this procedure, the animals usually died in from 5 to 16 days after inoculation, and the results left much to be desired. The problem was reinvestigated by Sawyer and Lloyd (1931) with the aim of making the test more delicate in operation, and after a series of experiments these workers succeeded in evolving the method next described.

The Intraperitoneal Mouse Protection Test

This test differs from Theiler's test since it utilizes the employment of another principle in its operation. Instead of introducing the mixture of virus and serum intracerebrally in a mouse, the animal is first injected intracerebrally with starch and thereafter intraperitoneally with a mixture of virus and serum to be tested. The action of the starch causes cerebral trauma which damages the blood brain barrier (see Friedemann and Elkeles, 1934) so that the virus can leave the circulation and freely invade the injured nerve cells. The time taken by the virus to reach the central nervous system from the general circulation proves an additional advantage as it affords ample opportunity for neutralization of the virus to occur—if antibodies are present—and at the same time it delays death of the mice by about 2 days. In consequence of this, if the serum injected possesses no neutralizing antibodies for yellow fever virus, the majority of mice succumb between the 7th and 11th days following inoculation. It has been mentioned that in performing the mouse protection test the virus-serum mixture has to be introduced intraperitoneally, and the necessity for employing this route of inoculation has been experimentally demonstrated by Sellards (1935 *a*), who showed that, although the brain of an animal was traumatized and massive doses of virus were administered subcutaneously, it failed to develop encephalitis. The tendency of the virus to enter the blood stream and brain is considerably greater when the material is injected intraperitoneally than by the subcutaneous route. A point worthy of mention is the fact that different strains of white mice are sometimes liable to vary in their susceptibility to yellow fever virus, and, accordingly, a suitable strain must first be procured for the purpose.

The value of the mouse protection test for yellow fever, the uniformity of its results, and the specificity of its response have now been universally accepted and accorded world-wide recognition.

Technique. The test is carried out according to the following method (Sawyer, 1931).

A mouse is injected intracerebrally with 0.03 c.c. of a solution consisting of 2 per cent. starch made up in 0.9 per cent. sodium chloride. Simultaneously, the animal is given an intraperitoneal inoculation of 0.2 c.c. of a 20 per cent. saline suspension of virus contained in mouse brain tissue, mixed with 0.4 c.c. of the specimen of serum being tested. Six mice are inoculated with each sample of serum and kept in a glass jar under observation for a period of 14 days. Each series of tests is controlled by including a known positive serum derived from a recovered human case of yellow fever, as well as a negative serum obtained from a person resident in a non-yellow fever country.

Before *interpreting the results*, death of all the 6 mice should occur in the case of the negative control serum not containing neutralizing antibodies for yellow

fever virus With the positive control, complete protection should result and all the mice should survive. Provided that the above controls are satisfactory, the results of the test serum may next be interpreted If all the mice remain alive, it can be assumed that the individual from whom the serum has been derived possesses antibodies for the virus and has, therefore, suffered from infection during the past. If, on the other hand, all 6 mice succumb to infection, then it can be concluded that the individual in question has never been infected with yellow fever virus during his or her life. It can also be argued that such nonimmune persons are likely to develop the disease in the
mediate to the above are ir
mice surviving, the greater
blood. Mahaffy, Lloyd, and
ards for the interpretation of results

TABLE 21

Number of Mice Surviving 10 Days after Inoculation	*Number of Mice Alive 4 Days after Inoculation*	*Interpretation of Ratio*
6	6	Positive
5	6	Positive
4	6	Inconclusive
3	6	Inconclusive
2	6	Negative
1	6	Negative
0	6	Negative
5	5	Positive
4	5	Positive
3	5	Inconclusive
2	5	Inconclusive
1	5	Negative
0	5	Negative
4	4	Positive
0	4	Negative

With less than 4 mice surviving on the 4th day the test is unsatisfactory (Whitman, 1943). A technique for testing small quantities of serum has been evolved by Lloyd and Mahaffy (1935).

The procedure requires only 0.3 c c of serum, and 2 animals can be injected intracerebrally with the serum-virus mixture. The method is said to give 95 per cent correct results

In Uganda the test employed by Smithburn (1945) utilizes 5–6 weeks old mice. A minimum of 0 3 c c of serum is necessary for the test. Groups of 6 mice each receive an intracerebral dose of 2 per cent. starch, and immediately afterwards an intraperitoneal dose of 0 6 c c of a mixture of 0 3 c c serum and 1.5 c.c. of 1 per cent. virus without preliminary incubation A procedure employing less than 0 3 c c of serum is also described If not more than one mouse dies the serum is assumed to contain protective antibodies. Should 2 or more mice survive serum is presumed to contain antibodies Death of one or more mice, or survival of more than 2, signifies an inconclusive result.

Intraperitoneal Test in Young Mice without Starch

The extreme susceptibility of very young mice to extraneural injection was shown by Theiler (1930) and Bugher (1941). Whitman (1943) devised a test employing 18 to 21 days old Swiss mice, some features of which are as follows The 50 per cent. end point of virus infectivity by the intraperitoneal route was accepted as 1 MLD and any immune serum providing complete protection against this dose was regarded as having a titer of 1 For example, it was observed that 0 02 c c. of 15 per cent mouse brain injected intraperitoneally in 21 days old mice had a titer of 10 MLD in that when diluted 1 10 it killed 50 per cent. of mice (Reed

and Muench, 1938). When the same material was titrated intracerebrally it contained more than one million MLD. When 2 parts of immune serum are added to 1 part of virus, the results of injecting 0.06 c c of the mixture into 18 to 21 day old mice are equivalent to injecting adult mice which have received intracerebral starch with 0 6 c c. This permits satsifactory tests in much smaller samples than required for the standard test For details of technique and dilutions used see Whitman (1943). At the end of 10 days the results are interpreted in the same manner as the standard test. Small amounts of antibodies are said to be detectable by this method. See also Loring for tests using young mice.

Specificity of Yellow Fever Virus Neutralizing Antibodies

Although a high degree of specificity is observed in the serological response following natural or artificially induced immunization against yellow fever virus in man, monkeys, and related primates, the same does not hold true for the cow, sheep, goat, dog, and other species. Koprowski (1946) adds that sera derived from the latter in Brazil also exhibited nonspecific neutralization against such a wide range of viruses as those of Japanese B encephalitis, St. Louis encephalitis, and West Nile virus, all of which presumably did not occur in Brazil. Furthermore, such antibodies could not be annulled by heating at 56° C

Natural Neutralizing Antibodies in Babies

Soper, Beeuwkes, Davis, and Kerr (1938) found that the presence of yellow fever antibodies in the sera of infants at birth was related to the antibody content of their mothers. Moreover, such immunity was fleeting in character and disappeared within the first few months of life.

Complement Fixing Reactions

The existence of specific complement fixing antibodies in the sera of human beings, and also in monkeys who have recovered from an attack of the disease, has been described by Moses (1929), Frobisher (1930, 1931, 1931 *a*, 1933), and Davis (1931 *a*) The method of preparing antigen and the technique of setting up the test is described on p. 104.

Antigens suitable for the test.

Frobisher (1931) reported satisfactory results using antigen extracted from the liver of infected monkeys by the use of isotonic and hypertonic salt solutions, combined with the application of alternate freezing and thawing. Saline extracts of monkey liver tissue tended to become anticomplemenatry when stored in cotton-stoppered flasks, therefore hermetically sealed ampules were essential Likewise, the addition of eosin or alcohol gave rise to similar anticomplementary properties The antigen was found to be destroyed by heating it to 55° C. for half an hour The addition of alcohol and alcoholic solution of cholesterin caused undue precipitation when added to the liver antigen, and the use of lipoids as sensitizing agents also was not to be recommended Either fresh liver tissue or that which had been desiccated *in vacuo* was found to react satisfactorily, although better results were obtained from the use of fresh liver Neurotropic strains of virus have also been found to act as efficient antigens, and in some cases virus contained in mouse brain tissue provided superior results to viscerotropic virus (Frobisher, 1933)

Other infective materials found to possess antigenic properties were monkey blood removed from the animal during the early stages of its illness, and also Berkefeld filtrates prepared from saline extracts made from infective mosquitoes, both of which reacted strongly in the presence of immune monkey serum In another paper, Frobisher (1931 *d*) reported that antigen prepared from the liver tissue of rhesus monkeys which had been freed from fat by treating the material in a Soxhlet extraction apparatus yielded results superior to that obtainable by any

other method. The use of such antigen for the complement fixation test is described on p 107.

Results. From his experiments Davis (1931) showed that the sera of monkeys which had recovered from yellow fever developed complement fixing antibodies No antibodies could be detected in the sera of normal monkeys or those which had been injected with dead virus, or passively immunized with monkey or human antiserum. The interesting point was also established that, in the case of monkeys which had survived an attack of yellow fever, after a lapse of time, when comple-

months later the test was still positive, although less pronounced, and a smaller amount of complement united From the results of their studies in Brazil, Soper and de Andrade (1933) concluded that a close correlation existed between the results of the complement fixation reaction, the mouse protection test, and the history of recent illness. Davis (1931) considered that the results of the complement fixation te low fever

man and monkeys

fo 17D virus has been

reported by Lennette and Perlowagora (1943) The latter mentions that since complement fixing antibodies disappear rapidly following infection, their presence in sera should be accepted as indicative of recent infection and as such should claim a place in immunity surveys when it is desired to ascertain the duration of infection. The test may also be of value in the early diagnosis of yellow fever (Perlowagora and Lennette, 1944)

The Precipitin Reaction

The technique of the test is described on p 103 During an acute attack of yellow fever in the monkey, a substance appears which can be precipitated from serum by treatment with the serum of an animal which has already recovered from the disease. This observation has been extended to human beings, and Hughes (1934) showed that the serum of a recovered patient contained a precipitin which reacted with precipitinogen reported in the blood of infected monkeys. This precipitinogen is not the virus of yellow fever, but is associated with the albumin fraction of the serum protein, and its amount in the circulating blood increases with the severity of the infection and declines with recovery (Hughes, 1934) It is possible that the presence of this substance in the sera of human cases of yellow fever may conceivably be of value as a laboratory aid to the clinical diagnosis and prognosis of the condition In discussing the relationship of the precipitin reaction to the complement fixation test, Hughes points out that parallel tests on monkeys have proved that in no case has a positive precipitin result been obtained without a simultaneous positive complement fixation response He adds that only 3 sera examined by him showed positive fixation of complement, without the simultaneous or preceding occurrence of precipitins The 2 phenomena, therefore, appear to be intimately related to one another in yellow fever

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On the shores of the Adriatic. The disease occurs in Dalmatia, Herzegovina, at the tributaries of the river Po, and Istria (see Gabbi, 1915 *a*, and Peschle, 1936).

In the Mediterranean area. It is commonly seen in Athens (Alivisatos, 1936), Malta (Whittingham and Rook, 1923 *a*), Haifa (Schapira, 1926), Sicily and Calabria (Gabbi, 1915 *a*), Corsica (Leger and Séguinaud, 1912), Jerusalem (Muhlens, 1912), as well as along the northern shores of Africa and Egypt (Sergent, 1914). It is being increasingly recognized in S France, e g, in Marseilles (Genevray, 1946), and Montpellier (Janbon, Harant, and Chaptal, 1942). It presented a definite military problem during the Second World War, incapacitating large numbers of men for up to 2 weeks (see, e g, Cullinan and Whittaker, 1943; Sabin, Philip, and Paul, 1944, Fleming, Bignall, and Blades, 1947).

Africa In Kenya cases have been observed by Jewell and Kauntze (1932), and at Dar-es-Salaam by Manteufel (1912).

In the Far East. The disease has been observed in China at Peking, Tientsin, and Hong Kong (Yang, 1934, Bolt, 1944).

South America. Cogollo Duque (1935) reported the outbreak of a disease resembling sandfly fever at Bolivar, Colombia

The Effect of Temperature on Seasonal Incidence

In India, the peak of the sandfly fever epidemic usually occurs in the spring of the year about May or April If, however, the temperature remains low, owing to the presence of late winter rains, then the onset of the epidemic is delayed until June, and this fact has been experimentally proved by Whittingham (1924), who showed that infected sandflies failed to transmit the disease to human volunteers when the temperature fell to 54° F. The early advent of warm weather in the spring, on the other hand, accelerates the onset of an outbreak.

Some Biological Characters of the Sandfly

In size the insects are about the dimensions of a pin's head (see Theodor, 1938). They breed among the cracks and crevices of walls, parapets, artificial embankments, and amid the rocks and stones of dried-up river-beds Gabbi (1915 *b*) states that in the volcanic districts of southern Italy old deposits of lava provide favorable conditions for their existence. The insects pass the winter months in the larval stage and breeding is encouraged by darkness, moisture, and traces of organic matter (see Smith, Mukerjee, and Lal, 1936). Favorite breeding sites for sandflies are the cracks and crevices found in the walls of dilapidated latrines Breeding also occurs in the boles of tree trunks, in consequence of which Gabbi (1915 *a*) has suggested that they may be transported from one place to another by sailing vessels. During the hot weather season, the insects assume their adult form and move from place to place by means of a series of short flights of about 50 yards each, and seldom at

... as they observed it during the summer months in Malta, occupied a period of about 8 weeks, depending upon the temperature, food supply, and humidity. The egg stage occupied 9 days, the larval stage 28, the pupal stage 10, and the imaginal stage 14 days Each phase of the life cycle of *P. papatasii* was shown to be governed by climatic conditions, the insect hibernated when the earth temperature fell below 65° F.; the optimum required for hatching of the imago was 70° to 90° F, and excessive moisture proved injurious to all stages of the developing insect Whittingham (1924) also found that the female of *P. papatasii* lived from 10 to 14 days, during which some fed daily, but others only at intervals of 3 to 4 days The time taken by an unfed fly to ingest a meal was about 2 minutes, and 5 to 10 minutes for one which had previously had a meal Wenyon (1913) induced

CHAPTER XLIV

SANDFLY FEVER

Synonyms

PAPPATACI fever, Phlebotomus fever, Three-day fever, Chitral fever, Summer fever, Mediterranean dengue, Simple continued fever, Heat apoplexy, *Influenza malarica*, "Dog disease," *Hundskrankheit*, *Sommerfieber*, *Sommerinfluenza*, *Soldatenfieber*, *Endemischer Magencatarrh*, *Febbre dei tre giorni*, *Mal della Secca*, *Febbre estiva*, *Gastroenteritis endemica*, *Gastroenteritis climatica*, and *Febbre da canape* (see Doerr and Russ, 1914).

Historical

The disease has existed for many years in Italy, where it has been known by different names Pick (1886) is credited with having been the first observer to describe the condition as a clinical entity, and Taussig (1905) as having first recognized its association with *Phlebotomus papatasii*. Although the disease is popularly referred to by English-speaking people as "sandfly fever," Newstead (1911) points out that as the pappataci flies belong to the genus *Phlebotomus*, this term is a misnomer and that the designation "phlebotomus fever" is more correct.

During the course of an epidemic among soldiers in Herzegovina, the malady received the attention of the Austrian military physicians, and in 1908 the commission consisting of Doerr, Franz, and Taussig (1909 *a*, *b*) proved it to be a virus infection transmitted by the sandfly, *Phlebotomus papatasii* (see also Doerr, 1910, 1911). The work of Doerr and his colleagues was soon confirmed by Birt (1910 *a*) and others, who proved that the etiological agent was a filter-passing virus Later Whittingham (1922, 1924) and Couvy (1922) isolated leptospira from suspected cases of sandfly fever, and for a time it was believed that the disease was caused by a spirochete and not a virus Following the researches of Kligler and Ashner (1928) in Palestine, it was clearly shown that leptospira could not be demonstrated in infected human blood or in sandflies captured in patients' houses, although a careful search was made for these organisms by means of direct microscopical, centrifugalization, animal inoculation, and cultural methods of examination. Emulsions prepared from infected and also uninfected sandflies have been examined by dark ground illumination for the presence of spirochetes by Poole and Sachs (1934), but without success In view of these negative findings, it is now agreed that sandfly fever is a virus disease and that the cases studied by those observers who found leptospira were in all probability some variety of Weil's disease

EPIDEMIOLOGY

Geographical Distribution

the climatic conditions are dry and arid,
r sandflies
rontier at Peshawar and Chitral, in the Central Provinces, and Northern Punjab At Chitral it was originally described as "Chitral fever" by McCarrison in 1906 A map showing the distribution of sandfly fever in different parts of India appears in the report of Megaw and Gupta (1927). (See also Young *et al*, 1926, Fawcus, 1930, 1933, Poole and Sachs, 1934, Anderson, 1939, 1941)

The U.S.S.R At Tashkent in Turkestan, cases have been reported by Raevsky (1929) and Petrov (1929 *a*), at Tiflis in Transcaucasia by Gridnew (1928), and in the Crimea by Petrov (1929 *b*) and Kremer (1930)

to suggest that *P. perniciosus* Newst., *P. minutus* Rondani, *P. sargenti* Parrot, and *P. caucasicus* Marzinowsky may be carriers of infection (see Viskovsky and Petrov, 1927, Newstead, 1911, Young, Richmond, and Brendish, 1926, Janbon, Harant, and [illegible]

1947).

CLINICAL FEATURES

The bite of a single insect is sufficient to infect a susceptible person (Birt, 1910 *a*). The site of trauma later becomes painful and tender and may show vesicle formation at its center, so that the lesion may superficially resemble that of chicken-pox.

With regard to the length of the *incubation period*, Castellani and Chalmers (1910) state that it may vary from a few hours to 5 days, but rarely 6 days, whereas Jewell and Kauntze (1932), who have studied cases in Kenya, maintain that it extends from 6 to 7 days but may vary from 3 to 9 days. Shortt, Poole, and Stephens (1934) found during the course of their experimental work that human volunteers who were bitten by infected sandflies developed the disease in 3 to 10 days' time. Volunteers who were experimentally inoculated with infected blood complained of symptoms after 3 to 4 days.

The *onset* is usually sudden and there may be prodromal symptoms. The disease starts with an initial chill or rigor which is accompanied by retro-ocular headache together with aches and pains in the muscles and joints of the back and legs.

Flushing of the face occurs and *the conjunctivae* become so markedly injected that they resemble the eyes of a bloodhound or mastiff, and hence the origin of the name *die Hundskrankheit*, or "dog disease," applied to it by the German writers (Taussig, 1905).

The *skin* is hot, dry, and irritable as the result of repeated scratching, and small vesicles and spots may appear on the fauces, tongue, and palate of the mouth.

Eye lesions such as conjunctivitis, neuroretinitis, and papilledema have been described by Jayle and Mastier (1935), Rameev (1936), and Shee (1942).

"Harara" is a skin condition produced by repeated bites and pruritus following the attacks of sandflies. It is commonly seen among newcomers to Palestine, and takes the form of an urticarial skin eruption confined to the face, arms, and legs. Small vesicles and papules about 7 mm in size develop in due course, and these become filled with clear or blood-stained fluid. At first the fluid is bacteriologically sterile, but later becomes invaded by organisms (see Doerr and Russ, 1909, Dostrowsky, 1925, and Szentkirályi and Lorincz, 1933).

Theodor (1935) reinvestigated the etiology of *harara*, and made the interesting observation that the skin reaction to the bite of the sandfly is a sensitization phenomenon which is acquired relatively quickly, and is later followed by desensitization. It has been claimed that the production of a wheal may localize virus, and protect against further invasion (Fleming, Bignall, and Blades, 1947).

The *temperature* is characteristic of the disease and usually rises to about 104° F. during the first 24 hours of the illness and thereafter declines, to reach normal 3 days later (see Fig. 37). Occasionally a terminal rise of temperature occurs (see McArthur *et al.*, 1930).

Pulse. A slow rate is frequently observed in sandfly fever, and Shortt *et al.* (1934) have stressed the value of bradycardia as a point of importance in the differential diagnosis of sandfly fever from other causes of pyrexia. The slow pulse may be present during the illness and persist throughout convalescence. Le Gac (1937) also mentions the value of bradycardia as a sign of diagnostic significance in the differentiation of pappataci fever from dengue, and adds that a slow pulse rate is due to cerebral hypertension.

sandflies in captivity to feed every second day, but Young, Richmond, and Brendish (1926) maintain that under ordinary conditions the female only feeds once, and is fertilized during the first 36 hours of adult life. After a meal it remains in houses for 60 to 78 hours, returns to its breeding ground, lays its eggs within 108 hours, and may repeat the process of feeding and oviposition a second time. Presumably, therefore, infection of the human being would occur during the second meal.

For fuller information regarding the life history and bionomics of *P. papatasii* the reader is referred to the work of Whittingham and Rook (1923 *a, b*) and Newstead (1911). Other data dealing with the conditions necessary for the breeding of different species of sandfly, their survival in nature, and captivity are contained in the researches of Marett (1915), Smith, Mukerjee, and Lal (1936), Raja (1936), and Smith, Mukerjee, and Halder (1936).

Experimental Work on *Phlebotomus papatasii*

Doerr and his coworkers were the first to prove that infected sandflies could be transported from an endemic area of the disease to a country in which it did not occur, and the infection reproduced in the latter, by permitting the insects to bite human volunteers This observation has received the independent confirmation of subsequent workers (see Birt, 1910 *a*, Whittingham, 1924; Shortt, Poole, and Stephens, 1934; and others), and the rôle of *P. papatasii* is established beyond all doubt Doerr, Franz and Taussig (1909 *a, b*) and Birt (1910 *a*) stated that insects which had fed less than 7 days before were noninfective to volunteers. A similar conclusion was arrived at by Shortt, Poole, and Stephens (1934), who found the period to vary from 6 to 8 days Reports indicate that the virus may survive for a time in the insect vector during its metamorphosis According to Doerr *et al.* (1909 *a*) it can be transmitted by an infected female sandfly through its eggs to the second generation. Whittingham (1924) suggests that this method is unlikely, and he has shown that the virus can be transmitted from one generation of sandflies to another in their breeding grounds by larvae which may have ingested the dejecta or dead bodies of their parents. The flies which acquired infection in this manner became infective to man, but only after they had received their first meal of blood Whittingham's findings have been contradicted by Young, Richmond, and Brendish (1926), who concluded from their own experiments that under normal conditions the female of *P papatasii* did not die in its breeding ground after oviposition Neither did the young larvae exhibit any preference for feeding upon the dead bodies of adult flies when other pabulum was accessible. Young and his colleagues maintain that an infection of the insect in its breeding ground must be postulated They dismiss the idea that this occurs by larvae eating the dead bodies of infected flies for the reasons mentioned above, and suggest that as about 4 per cent of sandflies are infested by acarian parasites (*Raphignathus yongi* Hirst, and *Trombidium hindustanicum* Hirst) These may act as intermediate carriers

Important work confirming the original contention of Doerr has been published by Mochkovski *et al* (1937). They showed that transovular transmission occurs, and that infection is transmitted to man by the bite of females of the second generation hatched from eggs laid by the parent 6–8 days after the infecting feed. Infection was produced by the bites of newly hatched sandflies, showing that no activation by a blood meal is needed

The position is still in doubt, however, for Sabin, Philip, and Paul (1944) infected larvae with virus, but did not find the adults to be infective, flies hatched from the ova of infected females were likewise noninfective

Other Possible Insect Vectors

Apart from *P papatasii*, very little is known about the species of sandfly concerned in the transmission of sandfly fever The presence of human cases, coupled with the existence of insects in proximity to patients, has prompted many observers

DISTRIBUTION OF THE VIRUS

The virus is constantly to be found in the blood in the early stages of the illness. Blood serum remains infective for some weeks if kept in the ice chest.

There is some difference of opinion regarding the duration of infectivity. Sabin, Philip, and Paul (1944) found virus in the blood 24 hours before and after the onset of fever, but not 40 hours after.

On the other hand, Shortt *et al.* (1940) found virus in the blood for a much longer time, up to 7 and sometimes up to 40 days or more

PROPERTIES OF THE VIRUS

Filtrability

Doerr (1908) proved that the causal agent of sandfly or pappataci fever was small enough to pass through the pores of a Reichel earthenware filter. Later, Birt (1910 *a, b*) filtered infective blood through a Chamberland F bougie, diluted it 1 9 in saline, and reproduced the disease in a volunteer with an inoculation equal to 0 7 c c. of undiluted blood.

Tedeschi and Napolitani (1911 *a, b*) and Graham (1915) used the Berkefeld filter, and Kligler and Ashner (1928), who employed the Berkefeld N candle, found that as little as 0.1 c c. of serum was enough to infect a susceptible individual Shortt, Poole, and Stephens (1934) reproduced sandfly fever in human volunteers by inoculating them with Chamberland L_3 and L_5 filtrates of infected blood They also produced a febrile reaction in *M. rhesus* monkeys with human blood which had been passed through the Chamberland L_{13} bougie

Size

Shortt, Pandit, and Rao (1938) used virus cultivated on the chorio-allantoic membrane and estimated the size at 160 mμ by filtration Sabin, Philip, and Paul (1944), however, suggested the virus was of about the same size as that of yellow fever.

Resistance to Physical and Chemical Reagents—Preservation

The virus is killed by heating to 55° C. for 10 minutes (Lépine, 1927), it has been stored for 7 days at 75 to 80° F. (Birt, 1910 *a*), and resists 2 per cent. sodium citrate and 50 per cent. glycerol for 14 days (Shortt, Poole, and Stephens, 1934) The virus withstands drying and can be preserved, by desiccating sera removed from patients on the first or second day of the illness, over sulfuric acid *in vacuo* (Shortt, Poole, and Stephens, 1936) It can be preserved for at least 6 months by

Cultivation in the Developing Egg

Shortt, Rao, and Swaminath (1936) have employed the original method devised by Woodruff and Goodpasture (1931, see Ch. XIII) for the cultivation of sandfly fever virus on the chorio-allantoic membrane of the chick embryo (see Rao, Pandit, and Shortt, 1936). Fourteen- to fifteen-day-old embryos were inoculated either

else with

the pres-

ions there

was a distinct thickening of the membrane, at the center of which was a dense opaque patch measuring about 10 mm in diameter. Examination of such material showed massive growth of inclusion material in the cells of the mesoderm; the

The disease in children has been studied by Peschle (1936), who states that the condition manifests itself as a mild febrile disturbance which may be accompanied by intestinal symptoms.

Nervous system. Trabaud (1930, 1931) attributes the aches and pains experienced in sandfly fever to the action of the virus upon the posterior part of the cord.

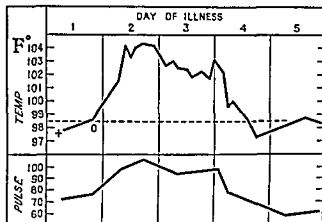


FIG. 37. Temperature chart in a case of sandfly fever.
(After Doerr, Franz, and Taussig, 1909)

Le Gac and Albrand (1937) examined the fluid in 14 cases of the disease and found that there was an increase in the pressure, albumin, and cell content, varying from 10 to 20 cells per c.mm The color of the fluid and its sugar content were normal and no cultivable bacteria were found (see also Le Gac, Samara, and Servant, 1939). It has been suggested that there is an increased blood-tissue permeability in sandfly fever (Gontaeva, 1943) Nervous symptoms may be sufficiently pronounced to simulate lymphocytic choriomeningitis (Pearson, 1941, Fleming, Bignal, and Blades, 1947)

Blood A leukopenia is the most characteristic change, and Whittingham (1922) has recorded that on the 4th day of the disease the total white cell count may fall to 3,500 per c mm Sabin, Philip, and Paul (1944) made a detailed study of the experimental disease. On the 1st day of fever, the total count was within normal limits, but there was a relative and absolute decrease in lymphocytes, and a relative and sometimes absolute increase in neutrophils due to an increase in immature cells. During the 2nd and 3rd days of fever, the number of lymphocytes began to return to normal At the same time the number of segmented neutrophils began to drop, and the immature cells increased to a point where they outnumbered the segmented cells. In certain patients, monocytes increased to constitute 16–40 per cent. of all leukocytes in the febrile and early postfebrile periods Leukopenia may be expected in 90 per cent of cases, chiefly at the end of the febrile period, and in the first 2 days of the postfebrile period

The urine is diminished in quantity during the febrile stage, but is otherwise normal in its constituents

The progress of convalescence varies in different cases. It may either be rapid and uninterrupted or else complications such as amnesia, mental depression, lethargy, bronchitis, or phlebitis may supervene Ocular changes have been described by Rameev (1936) Transverse furrows on the thumb and finger nails have been described (Mochkovski, 1937).

Mild attacks. Anderson (1941) recognizes a mild type of disease, seen on the northwest frontier of India.

IMMUNITY REACTIONS

Second and even third attacks of sandfly fever are not uncommon, even within one season (e g , Lambert, 1918, Janbon, Harant, and Chaptal, 1942) However, there seems little doubt that an attack does confer a certain degree of resistance, perhaps not evident for several months (McCarrison, 1915, Lambert, 1918, Lépine, 1927, Shortt, Poole, and Stephens, 1935, 1936, Livshitz, 1937, Sabin, Philip, and Paul, 1944, Cullinan, 1946)

The inhabitants of endemic areas almost certainly develop active immunity, presumably as a result of repeated infection, and, for example, it has frequently been found that newly-arrived European soldiers are more readily attacked than the local inhabitants.

Some attempts have been made at immunization by inoculation of live virus in man, and it is probable that some protection follows (Livschitz, 1937, Sabin, Philip, and Paul, 1944). The latter workers also used virus irradiated by UVL.

After inoculation of live virus, virus may be recovered from the blood, and neutralizing antibodies develop later (Shortt *et al*, 1940)

As first shown by Doerr *et al* (1909 *a*), virus neutralizing antibodies develop in convalescence in man Antibodies can be demonstrated by inoculation of virus-serum mixtures in monkeys, eggs, or even man

Virus neutralizing antibodies develop also in the sera of convalescent monkeys (Shortt *et al*, 1940)

PREVENTION OF SANDFLY FEVER

Advantage has been taken of the fact that the insects fly comparatively near the ground, and consequently individuals who sleep in the upper part of a house may escape being bitten In the case of military camps, removal to higher ground has had a similar effect (see Whittingham and Rook, 1923 *a*). Anderson (1939), however, reports that sandflies can fly up to 70 feet above ground, and that there is no evidence that persons in upper stories are protected, sandflies cannot fly against a wind of more than about 2 m p h The insects are attracted by light, and traps have been used for this purpose. Extrinsic factors such as atmospheric temperature and relative humidity have been found to affect the development of infectivity for man.

The following control measures have been recommended for military camps (*Bull U S Army med Dept*, 1945). The site should be on open, raised, dry,

shirts and full-length trousers must be worn Insect repellent on exposed parts of the body protects for 4–6 hours, dimethyl phthalate and pyrethrum are effective (Sabin, Philip, and Paul, 1944) For further information on the use of DDT, see Hertig and Fairchild (1948) and Semple (1948)

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cytoplasm of many cells appeared to have been wholly replaced by red-staining inclusion material but no characteristic inclusion bodies could be detected

These observations have been confirmed (Shortt, Pandit, and Rao, 1938, Shortt *et al.*, 1940, Demina, 1941 *a*, Khodukin, Soshnikova, and Kevorkova, 1943); the virus has been carried through numerous passages. The microscopical appearances are those of a proliferative necrosis, desquamation of the ectoderm, infiltration of the mesoderm, and proliferation of the entoderm (Demina and Levitanskaja, 1940). These authors reproduced the disease in man with a suspension of membrane.

Tissue Cultures

The virus has been cultivated in tissue cultures containing finely minced chick embryo, Tyrode, and human serum (Shortt, Pandit, and Rao, 1938, Shortt *et al.*, 1940). The culture virus was not pathogenic to mice or monkey. Demina (1941 *a*) also cultivated the virus.

HUMAN TRANSMISSION

In addition to those mentioned elsewhere in the text, a number of workers have transmitted the infection by injections of blood or serum or by the bites of infected sandflies (Anderson, 1941, Gontaeva, 1943, Sabin, Philip, and Paul, 1944, Fleming, Bagnall, and Blades, 1947). Sabin, Philip, and Paul passed the infection in series 7 times. The most effective route of inoculation was the intracutaneous or intravenous, the subcutaneous and intramuscular routes were less sensitive.

In Russia apparently sandfly fever is regularly transmitted in the treatment of schizophrenia (see Livshitz, 1937; Khodukin, Soshnikova, and Kevorkova, 1943)

ANIMAL INOCULATION EXPERIMENTS

Monkeys are mildly susceptible Tedeschi and Napolitani (1911 *b*) inoculated *Macacus cynomolgus* with 0 5 c c of a Berkefeld filtrate of human serum, and succeeded in reproducing a transient febrile illness After an incubation period of 7 days the temperature rose from 37 5° C to 38 2° C, and 3 days later it reached 39.3° C

More recently the work of Shortt, Poole, and Stephens (1934) has shown that the virus of sandfly fever is also feebly pathogenic towards *M rhesus* Thus, 5 out of 11 animals which were inoculated with 10 c c. of infected human blood developed a slight rise of temperature, attaining 103° F 3 to 4 days after inoculation The illness lasted from 1 to 5 days, and during this period the blood of one monkey proved infective to another animal of the same species It was also possible to reproduce a mild attack of sandfly fever in 2 human volunteers by injecting them with 5 c c. of infected monkey blood Later, Shortt *et al* (1940) recovered virus from the blood of monkeys, up to a maximum of 19 days after onset

Dogs, rabbits, and *guinea-pigs* have been inoculated with negative results (Tedeschi and Napolitani, 1911 *a*, Poole and Sachs, 1934) Apparently, Russian workers claim to have infected rabbits by "suboccipital" injection (see Khodukin and Shterngold, 1943) These workers could not infect mice intracerebrally.

Sabin, Philip, and Paul (1944) could not infect monkeys, mice, hamsters, rabbits, guinea-pigs, and cotton rats Birt (1910 *a*) also found that the virus was not pathogenic to laboratory animals

LABORATORY DIAGNOSIS

The virus survives in blood and serum for some considerable time, and samples may be mailed to the laboratory, where monkeys should be inoculated Of particular value is the susceptibility of the chorio-allantois (see Anderson, 1941)

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were no evident changes (Koprowski and Hughes, 1946, Laemmert and Hughes, 1947).

On intracerebral injection of a marmoset (*Callithrix jacchus*), virus circulated in the blood for 7 days, and the animal died, virus being recovered from the brain. Another animal of the same species, on intraperitoneal injection, showed virus in the blood for 5 days. Another species of marmoset (*Callithrix penicillata*) showed virus in the blood for 5–7 days after intracerebral or subcutaneous injection (Koprowski and Hughes, 1946).

It is evident that marmosets circulate virus in the blood for longer than do the other primates tested. This species is abundant in the forest at Pirataquissé, the locality from which Ilhéos virus was obtained.

Rodents

After intracerebral or subcutaneous inoculation, the following rodents showed virus in the blood stream for 2–5 days: *Oryzomys ratticeps*, *Proechimys dimidiatus*, white rats, guinea-pigs, and rabbits (Koprowski and Hughes, 1946). None of these animals showed any symptoms of encephalitis.

Marsupials

Four species of marsupial were tested by Koprowski and Hughes (1946), and showed considerable variation in response: *Metachirops opossum* and *Didelphis marsupialis* were not readily infected, *Caluromys philander* showed virus in the blood for 3 days after intracerebral injection, and after subcutaneous inoculation, *Metachirus nudicaudatus* showed virus in the blood for 8 days after intracerebral, and 9 days after subcutaneous injection. One of these animals died, and virus was found in the brain.

Other Animals

The domestic hen, the pigeon, and a local canary were tested by Koprowski and Hughes (1946). None developed encephalitis, and virus had disappeared from the blood in 3 days. Virus persisted in the brains of some canaries after intracerebral injection for at least 30 days. Virus was demonstrated in the serum of the leaf-nosed bat (*Carollia perspicillata*) for 6 days after intracerebral injection, following subcutaneous injection, vampire bats (*Desmodus rotundus*) showed virus in the blood for a maximal period of 3 days (Koprowski and Hughes, 1946).

EXPERIMENTAL TRANSMISSION BY MOSQUITOES

Groups of mosquitoes were infected by feeding on baby mice showing viremia. *Aedes aegypti* infected baby mice fairly readily. *Aedes serratus* and *Psorophora ferox* could also transmit the infection (Laemmert and Hughes, 1947).

On the basis of the results of animal experiments described above, Koprowski and Hughes (1946) suggested that the Ilhéos virus is maintained in nature by alternate passages between marmosets and mosquito vectors, with perhaps occasional passages to marsupials or rodents.

OTHER PROPERTIES OF THE VIRUS

Physical Properties

The virus is filtrable through Seitz EK and Berkefeld filters (Koprowski and Hughes, 1946).

Reaction to Physical and Chemical Agents

Virus can be preserved by freezing and dehydrating suspensions in human serum (Koprowski and Hughes, 1946).

The thermal death point lies between 60° and 65° C (Koprowski and Hughes, 1946).

CHAPTER XLV

THE VIRUS OF ILHÉOS ENCEPHALITIS

ISOLATION

The virus of Ilhéos encephalitis was isolated in the course of yellow fever studies from mosquitoes captured in the vicinity of Ilhéos in the State of Bahîa (Brazil) by Laemmert and Hughes (1947). Mosquitoes of the *Aedes-Psorophora* group were collected at Pirataquissé, located in forested country. An emulsion was injected at daily intervals for an 8-day period into a rhesus monkey. Serum was obtained from this animal at 3-day intervals, and injected into 21–27-day-old Swiss mice. Some mice showed signs of illness, and their brains were passed. The Ilhéos virus was isolated and readily maintained through 50 mouse passages.

There appears to be no doubt that the virus was isolated from the captured mosquitoes. Serum taken from the rhesus monkey before inoculation contained no antibodies neutralizing the virus, but serum removed about 10 weeks later contained such antibodies.

Another rhesus animal inoculated a month later with emulsions of *Aedes-Psorophora* mosquitoes from Pirataquissé developed antibodies after 21 days.

INFECTION IN MAN

The virus can set up an antibody response in man, but there is no evidence that it causes an overt infection (Laemmert and Hughes, 1947).

INFECTION IN ANIMALS

Mice

Virus was readily maintained by serial brain-to-brain passage in albino Swiss mice derived from the original Rockefeller stock (Koprowski and Hughes, 1946). The average survival time was found to be 6 days in early passages, and 4 days in the 40th passage, the incubation period decreased from 5–3 days. After intraperitoneal injection, virus invades the blood, but disappears completely by the 3rd day, there is, on the contrary, a rapid increase in virus content of the brain tissue, reaching a maximum at about the 3rd day, after which it remains at a uniformly high level. The virus can be found also in spinal cord, lungs, kidneys, adrenals, spleen, liver, and intestines, when these are tested 120 hours after intraperitoneal injection (Koprowski and Hughes, 1946). There is a marked loss in susceptibility to extraneural (intraperitoneal) inoculation in mice of 28 days or older, as compared to younger animals (8–21 days) (Koprowski and Hughes, 1946). Mice of 8 days old are almost equally susceptible to subcutaneous, intracutaneous, intraperitoneal, intranasal, or intracerebral injection (Koprowski and Hughes, 1946). Laemmert and Hughes (1947) found baby mice (3 day) particularly susceptible to subcutaneous injection. A proportion of adult mice who devour infected baby mice becomes infected. The infection is not spread by cage contact, as distinct from cannibalism (Koprowski and Hughes, 1946). The brains of infected mice, regardless of the route of inoculation, show encephalitis characterized by hyperemia, small foci of hemorrhage, and mononuclear infiltration (Koprowski and Hughes, 1946).

Monkeys

On intracerebral inoculation in *Macaca mulatta*, virus circulates in the blood from 3–6 days after inoculation, one monkey was noted to become paralyzed on the 12th day after injection, and virus was recovered from its brain, although there

CHAPTER XLVI

COLOMBIAN VIRUSES OF ROCA-GARCIÁ

Roca-García (1944) isolated 3 viruses in the course of studies on the epidemiology of jungle yellow fever in Eastern Colombia. Two viruses were isolated from lots of the *Anopheles* mosquito (*Kerteszia boliviensis*), and have been named "*Anopheles A*" and "*Anopheles B*" viruses The 3rd was isolated from *Wyeomyia melanocephala*, and was accordingly named "Wyeomyia" virus (it has also been called "*Sabethine I*"). There is no evidence that these agents are pathogenic for man

Anopheles A Virus

The virus passes through Seitz EK pads, and all grades of Berkefeld filter The virus can be preserved by drying from the frozen state, but not satisfactorily in glycerol. Virulence is lost in 30 minutes at 55° C.

On intracerebral inoculation, mice develop paralysis and die by 5 days On intranasal inoculation, virus invades and multiplies in the brain, it is also found in the lungs Adult mice are relatively insusceptible to peritoneal inoculation, but develop resistance even to intracerebral challenge. Adult mice are unaffected by subcutaneous injection, although developing a slight degree of resistance Baby mice are very susceptible to virus inoculated by this route. Virus was recovered from the blood of adult mice irregularly on the 2nd and 3rd days after injection, especially by the intracerebral route Virus is found in the brain, cord, and adrenals

Rhesus monkeys and guinea-pigs do not show symptoms, but may develop viremia

The following animals did not show signs of illness, but developed antibody: *Didelphis marsupialis*, *Metachirus nudicaudatus*, *Metachirops opossum*, *Dasyprocta*, *Dasypus*, and *Cabassous* Pigeons did not develop viremia, and antibodies did not appear

The virus has been carried through 10 serial passages in the fertile egg, inoculated directly into the embryo. It has also been cultivated in minced mouse embryo in Tyrode with 10 per cent human serum

Anopheles B Virus

The virus passes through Seitz EK pads and all grades of Berkefeld filter

Virulence is not retained for long when virus is dried from the frozen state, but may be preserved in glycerol Virulence is lost in half an hour at 55° C.

The symptoms in mice are different from those shown by animals infected by the other viruses The incubation period is about 5 days, and mice die a few hours after the onset of illness, without paralysis Adult mice can be infected nasally and cerebrally. Baby mice do not develop symptoms after subcutaneous inoculation. Viremia does not occur in mice infected with this virus

Guinea-pigs develop no illness and no viremia.

The virus has been cultivated in minced mouse embryo in Tyrode with 10 per cent human serum

Wyeomyia Virus

The virus passes through Seitz EK pads and all grades of Berkefeld filter.

The virus can be preserved by drying from the frozen state, but not readily in glycerol

Adult mice inoculated cerebrally or nasally, and baby mice inoculated subcutaneously, show the same type of reaction as to *Anopheles A* (above), except that the incubation is a little longer The virus is more widely distributed in the bodies

Virus loses infectivity in saline more rapidly than in 10 per cent. serum saline, in both media, activity is lost at the end of 20 days (Koprowski and Hughes, 1946).

Some infectivity is retained in infected brains in 50 per cent. neutral glycerol (Koprowski and Hughes, 1946).

Cultivation

The virus was readily cultivated in *tissue cultures* prepared from minced chick embryo (without CNS) in serum Tyrode (Koprowski and Hughes, 1946)

The virus was also cultivated on the chorio-allantois, and invaded the embryo (Koprowski and Hughes, 1946).

VIRUS NEUTRALIZATION TESTS

Technique

Virus neutralization tests can be performed by inoculating virus-serum mixtures intracerebrally in 22-day-old mice, but the method is not regarded as being sufficiently sensitive (Laemmert and Hughes, 1947). Antibodies are best demonstrated by inoculating virus-serum mixtures subcutaneously in 3-day-old mice, and observing for 15 days. Death usually occurs between the 5th and 10th days (Laemmert and Hughes, 1947).

Occurrence of Virus Neutralizing Antibodies

Virus neutralizing antibodies have been found in the sera of rhesus monkeys after cerebral or peritoneal inoculation (Koprowski and Hughes, 1946, Laemmert and Hughes, 1947), antibodies also develop in *Cebus versuta*, and marmosets (Koprowski and Hughes, 1946) Antibodies have been detected in rats, guinea-pigs, rabbits, various rodents, and marsupials (Koprowski and Hughes, 1946).

Antibodies were detected in the blood of a laboratory worker during the course of experiments He had had no illness (Laemmert and Hughes, 1947). Antibodies were also found in the sera of 3 persons resident in the Ilhéos area, and less strongly developed in the sera of 2 other persons (Laemmert and Hughes, 1947).

RELATIONSHIP TO OTHER VIRUSES

Antisera to the following viruses failed to neutralize Ilhéos virus significantly, when mixtures were tested subcutaneously in baby mice yellow fever, eastern, western, and Venezuelan equine encephalomyelitis, St Louis, Japanese B, and West Nile encephalitis, LCM, louping ill, Russian spring-summer encephalitis (Laemmert and Hughes, 1947).

In other tests, dilutions of various viruses were mixed with Ilhéos monkey antiserum Ilhéos serum did not neutralize *Anopheles A* or *Wyeomyia* viruses of Roca-García, Japanese B, or West Nile viruses (Laemmert and Hughes, 1947).

Ilhéos virus differs from *Anopheles B* virus, which is not infective for baby mice subcutaneously, Ilhéos infection of mice also differs clinically and histologically from that caused by Theiler's virus (Laemmert and Hughes, 1947)

Although precise tests have not been carried out, the properties of Ilhéos and Semliki Forest viruses appear distinct.

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CHAPTER XLVII

WEST NILE VIRUS

ISOLATION

This virus was isolated by Smithburn *et al.* (1940) from an African woman in December, 1937, in the West Nile district, Northern Province of Uganda. She had a temperature of 100.6° F. A sample of her blood was taken, and a virus was established in mice by cerebral inoculation.

DISTRIBUTION OF INFECTION IN MAN

Smithburn and Jacobs (1942) carried out an epidemiological survey, and examined the sera of 1,428 residents in 63 localities in the Anglo-Egyptian Sudan, Kenya, Uganda, the Belgian Congo, and Spanish Guinea. They found that the virus apparently had been active in the Sudan, Uganda, Kenya, and the Congo In the region where the West Nile virus was isolated in 1937, a survey carried out 2 years later showed that the sera of 16 per cent. of persons tested contained neutralizing antibody.

INFECTION IN ANIMALS

Mice

Smithburn *et al* (1940) found that the virus is infective by either the cerebral, nasal, or peritoneal routes The incubation period, by the cerebral route, may be only 2–3 days, when mice become hyperactive, later they become sluggish and weak, and paralysis of the hind legs may occur. Death takes place 12–72 hours after the first signs of illness

After cerebral or peritoneal inoculation, the virus enters the circulation and persists therein. Virus inoculated peritoneally reaches a high titer in the brain by the 3rd day.

Lesions do not occur regularly in the visceral organs, but are generalized in the brain There are perivascular lymphocyte infiltrations, and the cerebral nuclei and cortical pyramidal cells show the principal changes. Many nuclei appear vesicular. Some pyknotic degeneration of nerve cells is also present, especially in Ammon's horn The most outstanding change affects the pyramidal cells of the cortex, the cells of the cerebral nuclei, and the Purkinje cells of the cerebellum There is retraction of the processes, and slight shrinkage of the cells, which stain acidophil in cytoplasm and nucleus No inclusions are found

Working with mouse-passaged virus, it was shown that susceptibility to fatal infection by the cerebral route is unaffected by age, susceptibility to extraneural inoculation, however, decreases with increasing age of the animal, animals 21 days old being found refractory (Lennette and Koprowski, 1944 *a*, *b*)

Monkeys

[illegible]

be viremia. Antibodies develop in convalescence. After intravenous inoculation, there is fever without clinical signs of encephalitis, the animals develop antibodies and resistance (Smithburn *et al.*, 1940). The histological changes are similar to those in mice (see above).

On cerebral inoculation in *Cercocopithecus aethiops centralis*, the virus enters the circulation and induces the formation of antibodies.

of paralyzed mice than is the *Anopheles A*, being found in liver, spleen, lungs, and heart, as well as adrenals, cord, and brain.

No signs of infection follow inoculation of rhesus monkeys, *Didelphis marsupialis*, rabbits, or guinea-pigs

Cross Immunity and Cross Neutralization Tests

Intraperitoneal inoculation of all 3 strains rendered mice resistant to a cerebral challenge of the homologous agent, but not resistant to the heterologous viruses. The 3 viruses appear immunologically distinct. Mice immunized against *Anopheles A* showed a certain measure of protection against Theiler's virus (GDVII strain), but otherwise there was no indication that any of the mosquito viruses produced immunity to this agent.

Yellow fever immune sera did not neutralize the 3 viruses.

Immune sera to the following viruses did not neutralize *Anopheles A* virus eastern and western equine encephalomyelitis, St Louis and Japanese encephalitis, West Nile virus, and distemper virus.

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IMMUNITY REACTIONS

Antigen-Antibody Reactions

Most work has been carried out with the virus neutralization test. Virus neutralizing antibodies can be detected by the intracerebral inoculation of virus-serum mixtures in mice. Lennette and Koprowski (1944 *b*) have introduced a mouse protection test using the susceptible young mouse (up to 14 days of age). Virus-serum mixtures are injected subcutaneously in 3-day-old mice, or intraperitoneally in 8- or 14-day-old animals. The neutralizing power of antisera estimated by the extraneural routes is 38,000–78,000 times that shown by the cerebral routes

An antigen can be prepared from mouse- or hamster-infected brain that fixes complement with specific antisera, the infectivity of such antigens is destroyed by exposure to ultraviolet light (Havens *et al.*, 1943). Casals (1944) has also prepared a complement fixing antigen from infected brain (see also 1947).

Immunity in Man

The serum of the original case was found to neutralize the virus, when tested 3 months later; antibodies developed in the sera of 2 persons studying the virus, although they had no illness (Smithburn *et al*, 1940).

It is highly likely that subclinical, and probably clinical, infections occur over widely separated areas in Africa and give rise to serum antibodies (Smithburn and Jacobs, 1942).

Immunity in Animals

Active immunity.

Monkeys develop resistance to infection following inoculation with living virus (Smithburn *et al.*, 1940) Hamsters that survive infection become resistant to reinoculation (Havens *et al*, 1943). Mice can be actively immunized (Smithburn, 1942; Casals, 1944).

Serum antibodies in convalescence.

Experimental animals, recovered from infection with the virus, develop virus neutralizing and complement fixing antibodies. High titer sera for use in serological tests can be prepared in monkeys (Smithburn, 1942), guinea-pigs, or mice (Casals, 1944), rabbits (Lennette and Koprowski, 1944 *b*), or hamsters (Havens *et al*, 1943).

Nonspecific virus neutralization.

Koprowski (1946) found that the sera of certain species of marsupial and rodent captured in Brazil, notably *Didelphis marsupialis* and *Oryzomys*, in addition to neutralizing yellow fever virus, inactivated Japanese B, St. Louis, and West Nile viruses These latter agents are not known to occur in Brazil. This antiviral activity was believed to be due to the presence of some nonspecific substance unrelated to infection with yellow fever The inactivation could be demonstrated by both the intracerebral and intraperitoneal techniques The virus-inactivating substance was not destroyed at 56° C The quantity of the substance present in the blood fluctuated, but might be enough to inactivate a very high concentration of yellow fever virus.

RELATIONSHIP BETWEEN WEST NILE, ST. LOUIS AND JAPANESE B VIRUSES

This question has been investigated in cross resistance and cross neutralization tests, the CF technique has also been used.

1 Mice rendered resistant to St Louis or Japanese viruses are not resistant to West Nile virus (Smithburn, 1942) Mice rendered resistant to West Nile virus succumb to infection with St Louis or Japanese B viruses (Smithburn, 1942).

Infection in other Animals

No signs of illness develop in inoculated rabbits or guinea-pigs, but antibodies are stimulated (Smithburn *et al.*, 1940).

Hamsters

These animals are susceptible to virus inoculated cerebrally or peripherally (Havens *et al*, 1943; Watson and Smadel, 1943). The incubation period is 7–10 days. Some hours before death, the animals become spastic, exhibit coarse tremors and convulsions. Virus appears in the blood within 24–48 hours of intraperitoneal inoculation, and is demonstrable in the blood or spleen before the brain

PROPERTIES OF THE VIRUS

Filtrability

The virus passes through all grades of Berkefeld filter and Seitz EK pad (Smithburn *et al.*, 1940). The size of the virus has been calculated by filtration experiments at 21–31 mμ (Smithburn *et al*, 1940).

Reaction to Physical and Chemical Agents

The virus is readily preserved by drying from the frozen state (Smithburn *et al.*, 1940). It is destroyed by heating at 55° C. for half an hour It is slightly more resistant to heat when suspended in serum (Smithburn *et al.*, 1940).

Cultivation in Eggs

Virus multiplies readily in embryonated eggs inoculated by the chorio-allantoic, yolk sac, or allantoic routes (Watson, 1943). Embryos become sluggish and die in 2–5 days. Virus becomes distributed throughout the egg, although the allantoic fluid only contains small quantities Titrations can be performed by inoculation in eggs

Cultivation in Tissue Culture

Koprowski and Lennette (1946) studied the effect of prolonged cultivation *in vitro*. The virus was found to grow equally well in media containing mouse embryo brain, whole mouse embryo, whole chick embryo, or chick embryo without the CNS, the latter was adopted for routine use. Virus persisted in cultures for a very long time—at least 32 days. The lethal effects for mice and hamsters by the cerebral route were not altered during 79 transfers, although there was some prolongation of the survival time The continued propagation of the virus resulted in a marked attenuation of its ability to kill mice on extraneural inoculation Inoculated by the peritoneal route, in 21–28-day mice, decreased virulence was shown in the 26th passage, by the 50th passage, culture virus was practically avirulent under these conditions. At the 50th passage, virus killed only an occasional 14-day-old mouse, but was still fully virulent for 8-day and 3-day mice At the 68th passage, there was a decreased virulence for 8-day mice After 79 passages, the virus was found to be avirulent for hamsters by the peritoneal route

Lennette and Koprowski (1946 *b*) found that the 17DD High strain of yellow fever and the West Nile virus suppressed more or less completely the growth of influenza virus (PR8) in tissue culture

Experiments with Mosquitoes

The mosquito *Aedes albopictus* can become infected by feeding on hamsters inoculated with West Nile virus After an incubation period of some days, the mosquitoes can transfer the infection to other hamsters by biting (*Philip and Smadel*, 1943).

CHAPTER XLVIII

BWAMBA FEVER

THE CHARACTER of this disease was described by Smithburn, Mahaffy, and Paul (1941), Bwamba County lies in the Western Province of Uganda, 2300–2500 feet above sea level. It is partly heavily forested, and partly agricultural The natives live as family groups in clusters of huts close to their agricultural holdings During the course of the investigation, native labor was employed on building a motor road, and all persons suffering from fever of undetermined origin were bled. Nine individuals presented symptoms of the disease called Bwamba fever, and the same number of transmissible agents was isolated. All strains appeared identical

Clinical Features

The onset was sudden, with fever and frontal headache. Weakness and backache were present. Fever lasted for 2–5 days (average 101° F.), the headache and backache for 2–3 days longer. Convalescence was rapid and complete The pulse was strikingly slow, averaging 84

Eight of the patients were nonresidents of the area, and had worked in the heavily forested area for 13–83 days before onset.

Possible intermediate animal hosts were numerous, but possible arthropod vectors were not troublesome.

Infection in Mice

Mice are susceptible to intracerebral or intranasal inoculation, but not to intraperitoneal or subcutaneous injections The incubation period varies from 4–6 days according to the dose and route. The first sign of illness is marked hyperactivity, often with exophthalmos Next the animal becomes normally reactive or sluggish, and presents photophobia. Paralysis of the hind quarters is not usual. Virus may be detected in the blood from the 4th–8th day after inoculation.

Microscopically, there is moderate to marked vascular dilatation and congestion of the cerebral blood vessels. Small hemorrhages may occur. Widespread and diffuse degenerative changes occur in the cortical pyramidal cells The nuclei are swollen and foamy or vesicular, with marked derangement of the chromatin pattern Many cells show small round or oval single or multiple intranuclear inclusions. Inflammatory changes are minimal

s inoculated by the
use, however, when
age
with the growth of
Bwamba virus injected by the same route (Vilches and Hirst, 1947).

Infection in Other Animals

The virus causes fever, without other significant signs, on inoculation in rhesus monkeys, and elicits the formation of antibodies. Viremia may occur shortly after inoculation

Rabbits develop no illness following cerebral or corneal inoculation Young guinea-pigs inoculated cerebrally show an irregular febrile response, and develop antibody

Filtrability and Other Properties

The virus passes Seitz EK and Berkefeld filters. Ultrafiltration experiments suggest a size of 113–150 mμ.

Casals (1944) likewise found no evidence of cross resistance in active immunity experiments, and the same conclusions were reached by Lennette and Koprowski (1946 a).

2. However, in neutralization tests with antisera, there is definite cross reaction between the 3 agents, and this complicates the serological diagnosis. Thus St. Louis antisera cross neutralize West Nile virus (Smithburn and Jacobs, 1942, Casals, 1944). Japanese B sera neutralize St. Louis and West Nile viruses (Smithburn and Jacobs, 1942; Casals, 1944). Smithburn (1942) reported that St. Louis and Japanese sera cross neutralize West Nile virus, but the neutralization is less effective than that brought about by the homologous serum. West Nile sera (human, mouse, or monkey) do not neutralize the other 2 viruses (Smithburn, 1942; Casals, 1944) However, Casals (1944) found that high titer West Nile sera prepared in hamsters or guinea-pigs do neutralize Japanese B virus.

The above observations made in adult mice have been repeated by Lennette and Koprowski (1946 a) using the sensitive extraneural test in 3-day-old animals. They found that St. Louis sera neutralized West Nile and Japanese B viruses, Japanese B sera neutralized St Louis and West Nile viruses, West Nile sera neutralized St. Louis but not Japanese B virus

3. Using complement fixing antigens prepared from infected brain tissue, Havens *et al.* (1943) could not demonstrate any cross reaction between the 3 viruses.

Casals (1944), also using complement fixation tests, found that Japanese serum reacted with both St. Louis and West Nile antigens, St. Louis serum reacted with Japanese antigen, and West Nile serum with Japanese antigen

We may conclude that the 3 agents are immunologically distinct, but share small amounts of one or more common antigenic components

RELATIONSHIP TO OTHER VIRUSES

West Nile, Japanese B, and St. Louis viruses are unrelated antigenically to the viruses of Western equine encephalomyelitis (Casals, 1944). West Nile virus is probably related antigenically to the Russian encephalitis virus and louping ill (see Ch. XCVIII) West Nile antisera do not neutralize *Anopheles A* virus (Roca-García, 1944), or the virus of Bwamba fever (Smithburn, Mahaffy, and Paul, 1941). The virus is not neutralized by yellow fever antisera (Smithburn *et al*, 1940) It is distinct from Bunyamwera virus (Smithburn, Haddow, and Mahaffy, 1946).

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CHAPTER XLIX

SEMLIKI FOREST VIRUS

THIS VIRUS was isolated from *Aedes abnormalis* mosquitoes caught in a forest in Bundinyama, Bwamba, about 1½ miles from the southern boundary of the Semliki Crown Forest (Smithburn and Haddow, 1944). It was established by intracerebral inoculation in mice.

Pathogenicity for Man

No definite illness has been attributed to this virus, but as antibodies have been detected (see below) it is probable that it is pathogenic. It would presumably be transmitted by *Aedes* mosquitoes, and a reservoir in forest monkeys such as *Cercopithecus nictitans* is probable

Infection in Mice

to 10^{-10} for intracerebral inoculation, to obtain an end point; a final dilution of 10^{-9} usually suffices to detect an end point by abdominal inoculation. The virus is also pathogenic for mice by subcutaneous inoculation, in about the same concentration as by the abdominal route.

Clinically, the first appearance of illness is paralysis in the hind limbs The mice become prostrate, and death not infrequently occurs during convulsions. With large doses of virus, all mice may be dead within 48 hours, with smaller doses, death may be delayed for 5–7 days.

Virus is found in the kidneys in greater titer than in other abdominal or thoracic viscera.

The brain shows hyperemia with small hemorrhages in some animals. There is also some perivascular cuffing with lymphocytes, and sometimes lymphocytic infiltration of the meninges or choroid, or both Microscopic foci of infiltration are found surrounding necrotic nerve cells, especially in the medulla.

Infection in Guinea-pigs and Rabbits

Guinea-pigs and rabbits can be infected intracerebrally with virus of enhanced virulence, but do not succumb from the effects of extraneural inoculation (Smithburn and Haddow, 1944).

Infection in Monkeys

A rhesus monkey inoculated cerebrally with late passage mouse virus developed fever and paralysis, and was dead after 4 days (Smithburn and Haddow, 1944). These workers also investigated the response of wild monkeys. A red-tail monkey (*Cercopithecus nictitans*) was febrile 3–9 days after subcutaneous inoculation, and showed viremia 3 days after inoculation, inoculated cerebrally, this monkey developed encephalitis A blue monkey (*Cercopithecus mitis* Wolf), inoculated cerebrally, developed fever in 48 hours and had viremia. A grivet monkey, inoculated cerebrally, developed encephalitis, but recovered slowly.

Filtrability and Other Properties

The

burn anc

reactions

The virus can be preserved readily by drying from the frozen state It is destroyed by heating at 50° C. for 30 minutes. Serum gives some protection to the virus against the effects of heat and saline solution.

Virus Neutralizing Antibodies

Virus neutralizing antibodies can be detected by inoculating serum-virus mixtures intracerebrally in mice 6–8 weeks old.

Normal human, monkey, rabbit, and guinea-pig sera were not found to neutralize the virus of Bwamba fever.

The virus was not neutralized by antiserum to West Nile virus. It is distinct from Bunyamwera virus

Acute phase sera did ion occurred in convalescence. illness and his convalescent serum neutralized the virus.

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CHAPTER L

BUNYAMWERA VIRUS

THIS VIRUS was isolated in Entebbe from a batch of over 4,000 *Aedes* mosquitoes caught in the uninhabited Bunyamwera III district of the Semliki Forest, Bwamba County, Western Uganda, by Smithburn, Haddow, and Mahaffy (1946). The virus was isolated by inoculating a rhesus monkey and was maintained by intracerebral passage in mice.

Pathogenicity for Man

Although not proved, it seems probable that this virus is pathogenic for man. Smithburn, Haddow, and Mahaffy (1946) made the following observations: 53 sera from persons residing on the edge of the Semliki Forest were examined prior to the isolation of the virus, but none contained antibody. The sera of a further 298 residents in Bwamba County were tested about a year after the isolation of the virus, and 10 per cent. contained protective antibodies. The serum of one patient with a suspected virus encephalitis also contained protective antibody.

There may be a reservoir of the virus in monkeys, as the serum of a colobus monkey has been found to contain antibody. Virus could be transmitted to man by *Aedes* mosquitoes.

Infection in Mice

Mice can be infected with the virus (Smithburn, Haddow, and Mahaffy, 1946). The first sign of infection is hyperactivity and hyperreactivity. The animal responds to stimuli by leaping or racing, and runs in circles. Convulsions usually precede death. The animal may survive the hyperactive stage and become paralyzed. Convulsions may occur in this phase also. With 14th passage virus inoculated intracerebrally, the mean survival time in mice receiving 6750 MLD was 3.3 days, with 6 M.L.D. it was 6.5 days. The virus infects mice also by the intraperitoneal, intranasal, and subcutaneous routes. It was transmitted in series only by the intracerebral route. Virus is found in the blood at the time of onset of objective illness. There is more virus in the spleen than other thoracic or abdominal viscera. The lesions produced by the virus are insignificant. The brain shows congestion and hemorrhages in the lateral ventricles. Degeneration of nerve cells is present, especially in Ammon's horn and basal nuclei.

Infection in Monkeys and Rabbits

The virus is not invariably fatal to rhesus monkeys by the subcutaneous route. During a febrile response, virus is found in the blood. Rabbits hyperimmunized with live virus showed no illness, but antibodies developed (Smithburn, Haddow, and Mahaffy, 1946).

Filtrability and Preservation

The virus passes through Seitz EK pads, and all 3 grades of Berkefeld filter. It can be preserved by desiccation from the frozen state, or by storage in glycerol (Smithburn, Haddow, and Mahaffy, 1946).

Virus Neutralizing Antibodies

Pro[illegible] [illegible]tion of mice cerebrally or periton[illegible]. Antibodies were found in the ser[illegible] wild monkey (see above).

loses potency in saline at low temperatures, but retains potency for several weeks in serum in the refrigerator. It survives heating at 60° C. for ½ hour, but not 1 hour. It is inactivated in ½ hour at 62° C.

Cultivation in the Egg

Virus has been successfully propagated through 33 serial passages in the embryo chick (Smithburn, 1946). The series of passages was initiated with a filtrate prepared from an infected mouse brain injected into the region of the developing embryo. Eggs incubated for 7–9 days prior to inoculation were employed. Passage was performed with a filtrate of ground up embryo, injected into the region of the embryo. Virus multiplied readily, and embryos succumbed between the 11th and 24th hours after inoculation. Tests for the presence of virus were made by inoculating mice intracerebrally. The titer of virus in embryonic chick tissue varied between 1 in 3.65 million and 1 in 210 million, and there was no decline on passage. Virus could be detected in the developing embryo from about the 4th hour of incubation onwards The virus content seemed to remain more or less stationary after the death of the embryo The curve of multiplication proceeded, up to 12 hours, by logarithmic progression. Beyond that point, there was a flattening of the curve but a further absolute increase. Macroscopically the embryos showed varying degrees of hyperemia and hemorrhage. Microscopic examination showed dilatation and congestion of vessels and extravasation, especially in the subcutis. Necrosis of cells only occurred to a slight extent and was not marked in any special tissue

Immunity Mechanisms

Antibodies can be demonstrated by inoculation of mice intracerebrally or intra-abdominally with virus-serum mixtures, the abdominal route is preferred (Smithburn, Mahaffy, and Haddow, 1944).

Individuals residing in 4 widely separated areas of Uganda possessed protective antibody Antibodies were also found in 6 primates, especially in *Cercopithecus nictitans* (Smithburn, Mahaffy, and Haddow, 1944).

Antibodies develop in the sera of rabbits, guinea-pigs, and monkeys inoculated with virus (Smithburn and Haddow, 1944)

Differentiation from Other Viruses

Semliki Forest virus has been differentiated from the viruses of yellow fever, Bwamba fever, St Louis and Japanese B encephalitis viruses, eastern and western equine encephalomyelitis, and West Nile virus (Smithburn, Mahaffy, and Haddow, 1944) It is distinct from Bunyamwera virus (Smithburn, Haddow, and Mahaffy, 1946)

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CHAPTER LI

COLORADO TICK FEVER

THIS DISEASE has been recognized in the Rocky Mountain areas of America since the end of last century, and has been called American mountain fever (Toomey, 1930–1) or Colorado tick fever (Becker, 1926, 1930). Modern interest dates from these papers of Becker and Toomey which clearly established the disease as an entity distinct from Rocky Mountain spotted fever. Topping, Cullyford, and Davis (1940) carried out an important study in Colorado. A general review is that of Florio and Stewart (1947), and Toomey gives an extensive bibliography to the earlier work.

CLINICAL FEATURES

A number of authors has described the clinical features which are very characteristic (e g, Becker, 1930, Toomey, 1930–1; Shaffer, 1935; Topping, Cullyford, and Davis, 1940, Collins, 1944; Florio and Stewart, 1947).

The incubation period is probably from 4–5 days Prodromal symptoms are rare and the onset is usually sudden The following symptoms are commonly observed: headache, chills, generalized myalgia, especially in back and limbs, photophobia, anorexia, and nausea. The throat and conjunctivae may be slightly congested.

The temperature soon reaches 102–104° F., where it remains for 48 hours The patient then apparently recovers for another 48 hours and the temperature is normal, but fever usually recurs. The second bout lasts for a further 2–3 days Convalescence is rapid, and fatalities or complications are unknown. There is not usually any marked reaction at the site of the tick bite, but an indolent ulcer may develop later.

Leukopenia is marked, and the total count may go down to 2000–3000 per c.mm. (Topping, Cullyford, and Davis, 1940, Collins, 1944; Florio, Stewart, and Mugrage, 1944, Florio and Stewart, 1947). All the leukocytes are absolutely reduced, except the monocytes, and there is a "shift to the left" in the polymorph series. On the 3rd–4th day after clinical recovery, the majority of cases shows an occasional large lymphocyte with basophilic bodies in the cytoplasm. These are spherical and measure about 0 5μ; they occur singly and in pairs and resemble nuclear material (Florio, Stewart, and Mugrage, 1944).

The virus is present in the serum of those naturally or experimentally infected, and can be recovered in the first or second bout of fever.

EPIDEMIOLOGY

The disease occurs mainly in Colorado, where there are 2 particular foci, one with the town of Boulder as center, and the other on the western slopes with Delta as the center, the flat prairie part of the state is free, but the disease has been reported from practically the whole mountainous region, it occurs also in Utah, Idaho, and Wyoming (Topping, Cullyford, and Davis, 1940)

The infection is contracted from the bite of ticks, and patients practically always give a history of being bitten by the wood tick *Dermacentor andersoni* Stiles, 4–6 days before the onset of illness The infection is transmitted transovarially in the tick, to at least the next generation of adults (Florio and Stewart, 1947). As in the case of Rocky Mountain spotted fever, the disease is commoner in adult males, who from their occupations are more likely than others to be exposed to ticks. The disease has a definite seasonal incidence, being found in spring and early summer before the hot, dry weather causes the ticks to disappear.

Colorado tick virus or a closely similar agent has been isolated from *D. variabilis* Say obtained from Long Island (Florio and Miller, 1948).

Differentiation from other Neurotropic Viruses

Tests were carried out with mixtures of Bunyamwera virus and antisera against other neurotropic viruses inoculated intracerebrally in mice Bunyamwera virus was found to be distinct from the following viruses. yellow fever, Bwamba fever, West Nile, St. Louis or Japanese encephalitis, eastern or western equine encephalomyelitis, or Semliki Forest (Smithburn, Haddow, and Mahaffy, 1946). Nor was any relationship shown to the viruses of Rift Valley fever, horse sickness, or to "Lot 6" virus

"Lot 6" Virus

This virus was isolated in the Entebbe yellow fever laboratory, but has not yet

REFERENCE

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RELATIONSHIP TO OTHER INFECTIONS

Rocky Mountain Spotted Fever

Both diseases are tick spread, and occur in the same area, although RMSF has a wider distribution. Colorado fever is contracted in the upper timber reaches of mountain valleys, at an altitude above that where RMSF is endemic (Toomey, 1930–1). The two diseases are, however, quite distinct for the following reasons (Toomey, 1930–1, Florio, Stewart and Mugrage, 1944; Florio, Mugrage and Stewart, 1946) The clinical course of Colorado fever is different, with its intermittency, mildness, and rapid convalescence It is possible to contract both infections. Volunteers vaccinated against RMSF could still be infected with Colorado virus. Colorado immune serum did not fix complement with RMSF antigen (DeBoer *et al*, 1947).

Dengue

Clinically, the disease resembles dengue fairly closely, especially as regards the "saddleback" temperature, leukopenia, and short course, but there is no rash in Colorado fever. Reciprocal challenge tests performed on human convalescents have shown that an attack of one disease confers no protection against the other (Florio, Mugrage and Stewart, 1946, Florio *et al*, 1946, Pollard *et al*, 1946 *a*)

Other Viruses

Colorado immune mouse serum did not fix complement with antigens prepared from the following agents (DeBoer *et al*, 1947): eastern and western equine encephalomyelitis, murine typhus, American Q fever, St. Louis and Japanese encephalitis, or rabies

Cross neutralization tests in mice showed no relationship between Colorado virus and those of louping ill or Russian spring-summer encephalitis (Koprowski and Cox, 1946), nor was any relationship shown with Venezuelan, eastern or western equine encephalomyelitis, Japanese, St. Louis, LCM, yellow fever, dengue, or California viruses (1947 *a*).

Challenge experiments in man detected no relationship between Colorado and Bullis fevers (Pollard *et al*, 1946 *b*).

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PROPERTIES OF THE VIRUS

To date, the observations point to *antigenic homogeneity of the various strains* (DeBoer *et al.*, 1947).

Experimental Transmission

The disease can be transmitted to *man* by the inoculation of human serum or virus passed experimentally, and the infection can be propagated in series (Florio, Stewart, and Mugrage, 1944, 1946, Florio *et al.*, 1946; Pollard *et al*, 1946*a*, Black, Florio, and Stewart, 1947). The experimental disease closely resembles the natural infection.

Hamsters are susceptible to intraperitoneal injection of human serum, and the infection can be passed in series using hamster serum (Florio, Stewart, and Mugrage, 1944, 1946; Koprowski and Cox, 1946; Black, Florio, and Stewart, 1947). There are no outward signs of illness, but the animals develop leukopenia, and round blue bodies up to 0.5μ in diameter may be found in the cytoplasm of atypical large lymphocytes Histologically, significant lesions occur only in the spleen (Black, Florio, and Stewart, 1947). Lesions are maximal on the 3rd day after inoculation They take the form of alterations in cellular type, and in the arrangement of the follicular lymphoid tissue, as well as a partial or complete disappearance of the normal well-defined follicular margin.

Mice can be infected. Koprowski and Cox (1946) established the virus in brown agouti and albino Swiss mice, using the cerebral route. Symptoms develop in 3–4 days, when the animals become hyperexcitable, and have ruffled fur, paralysis is rare. Virus can be found in the blood, but the maximum amount is found in the brain and cord The virus has also been transmitted to 21-day Swiss albino mice by the cerebral route by DeBoer *et al.* (1947). Eight-day mice are susceptible by the abdominal route (Koprowski and Cox, 1947 *a*)

Size, Filtration, and Cultivation

Florio, Stewart, and Mugrage (1946), on the basis of experiments in hamsters with collodion membrane filtrates, concluded that the agent passes through a membrane of 24 mμ pore diameter The size was estimated at 35–50 mμ by Koprowski and Cox, (1947 *a*) The virus passes Berkefeld N and Seitz EK filters (Koprowski and Cox, 1946).

Koprowski and Cox (1946) found that on yolk sac inoculation, virus generalized and could be recovered from the embryo, serial transfers could be carried out (see also 1947 *b*)

IMMUNITY

Active Immunity

One attack of the disease in man appears to immunize against a further infection Volunteers who contracted the disease 9–11 months before experimental infection, developed no, or only transient, symptoms on experimental inoculation (Florio, Stewart, and Mugrage, 1944)

Mice that survived inoculation with early passage virus were found to be resistant to challenge with active virus (Koprowski and Cox, 1946). Egg-adapted virus could also be used (1947 *b*)

Serum Antibodies

Complement fixation can be demonstrated between infected tissues and human or animal antiserum The antigen is best prepared by lyophilization of mouse brain and extraction with benzene, toluene, or dichlor-ethylene (DeBoer and Cox, 1946, DeBoer *et al*, 1947). In man, antibodies probably appear about 2 weeks after onset, and persist for as long as 3 years (DeBoer *et al*, 1947)

Hyperimmune sera, e g, for use in complement fixation tests can be prepared in mice by a course of peritoneal injections (DeBoer *et al*, 1947).

Gover, Reed, and Collins (1934) found that an undue incidence of respiratory conditions was associated with a subnormal mean temperature, especially in the autumn. To a lesser extent, the incidence of these conditions (at that season) was associated with a raised relative humidity, a lowered temperature range, and lessened s[illegible] weather during the warm season [illegible] than were changes in the cold season [illegible] raised the incidence of colds, namely: temperature range, morning dry bulb temperature, evening dry bulb temperature, [illegible] pressure.

2. *Other* [illegible] poor ventilation (Kler, 1945), [illegible] espert, 1944), food allergy (Coca, 1941, Brown *et al*, 1943), chronic [illegible] e, e g, Locke, 1939, Stock, 1946), the onset of menstruation (Kler, 1945) In industrial workers, colds are more prevalent in office than factory employees (Kler, 1945). Locke (1939, 1940) finds the incidence higher in those who react abnormally to tests involving the breathing of O_2 or 5 per cent CO_2.

CLINICAL FEATURES

We do not propose to waste time in describing in detail the straightforward clinical features of the common cold, which are so well known that only certain aspects need be considered. Thus, Townsend and Sydenstricker (1927), in the course of an extensive survey of respiratory infections in the United States of America, recorded the following relative incidence of symptoms running nose, 81 per cent., obstruction of nostrils, 44 per cent; sudden onset, 37 per cent; cough, 31 per cent., headache, 19 per cent, sore throat, aches in body or limbs, each 14 per cent; fever, 13 per cent, inflammation of eyes, expectoration, each 12 per cent.; constipation, 10 per cent, tightness of chest, 4 9 per cent., and chilliness, 3 4 per cent. Similarly, Van Volkenburgh and Frost (1933) found that nine-tenths of their cases had coryza, two-thirds a cough, half a sore throat, a quarter were confined to bed, and a quarter were febrile X-ray examination may detect transient pulmonary shadows (Kennedy, 1943).

Complications The common cold, itself harmless, may nevertheless give rise to various disorders which have serious consequences, such as sinusitis, otitis media, mastoiditis, and in consequence meningitis, acute bronchitis and bronchopneumonia, chronic bronchitis which may lead to emphysema and bronchiectasis, tonsillitis, and enlargement of the cervical glands Recording the complications of 1,388 cases of minor respiratory disorder, Van Volkenburgh and Frost (1933) obtained the foll[illegible] *per cent*, *adenitis*, 4 6 *per cent*, *sinusitis*, 2 8 per cent, oth[illegible]

[illegible] *re unlikely* to [illegible] statistics in support may be quoted Thus, [illegible] ry of some 2,500 American students He found that 23 per cent. had at least 4 colds a year, 60 per cent had 2 or 3, while only 17 per cent had not more than a single cold. For 2 years 484 persons were examined by Van Volkenburgh and Frost (1933). They found that the average was 3 attacks per person of minor respiratory disease. Of 685 adult male students studied by Townsend (1924) only 71 escaped a cold during the 5½ months of winter Smiley (1944) estimated the number of colds (without fever) in U S Naval personnel at 2500 per 1000 men

PATHOLOGY

[illegible] (1930) examined a number of biopsy specimens of nasal mucosa. In the [illegible]

CHAPTER LII

THE COMMON COLD AND MINOR RESPIRATORY ILLNESSES

KRUSE (1914) suggested, on the basis of experimental work, that the causal agent of the common cold was a filtrable virus Although the disease has all the appearances of a virus infection, little further progress was made until Dochez and his associates showed that the chimpanzee could be infected. Dochez's virus has not been widely isolated, and it appears possible that it is not the usual causal agent.

The clinical picture of the common cold is often characteristic enough, especially when afebrile; the term "nasopharyngitis, acute, catarrhal" may usefully be used (see Faust and Simmons, 1944). Trouble arises in differential diagnosis when the catarrhal symptoms are complicated by pyrexia ("feverish cold"). The term "*catarrhal fever*" *is often used for such cases, but this condition certainly includes* many infections that are not caused by the common cold virus (Ludwick and Jones, 1943, U.S. Naval Research Unit No. 1, 1944).

There is no doubt that a number of agents gives rise to symptoms and signs resembling those of the common cold. Thus, the Commission on Acute Respiratory Diseases (1947 *a*, *c*) *distinguishes between 5 mild respiratory illnesses as follows.*

1. The term "undifferentiated acute respiratory disease" (ARD) is applied to an acute febrile disease of short duration characterized by constitutional reactions or local respiratory symptoms, sufficiently severe to require hospitalization, and by epidemic occurrence in recruits in the winter. Diagnosis is made largely by exclusion of more obvious causes.

2 The features of "severe common cold" (SCC) are coryza, constitutional symptoms, and fever.

3. "Common cold" (CC) is characterized by coryza with little or no fever

4. "Bronchitis resembling atypical pneumonia" (Br-AP), is distinguished from atypical pneumonia (AP) by absence of consolidation

5. "Minor respiratory illness" (MRI) refers to a mild, usually nonfebrile, respiratory illness observed among recruits.

In addition, it is probable that the clinical picture of the common cold can arise in infection with viruses such as influenza and atypical pneumonia, and bacteria may also be responsible

A number of useful reviews of the literature are available (Thomson and Thomson, 1932, Browning, 1935, 1942, Stuart, 1938, Greenspan, 1943)

PREDISPOSING CAUSES

1. *The weather* While some have found that climatic conditions *per se* have little, or at most only very slight, effect, van Loghem (1928–9) has stressed the role of variations in the air temperature, falls increasing, and rises decreasing, the incidence of colds. Milam and Smillie (1931) found that a slight, but abrupt, drop in the atmospheric temperature coincided with the development of an epidemic Similar observations have been made by Brown *et al* (1945), and Kler (1945)

Many stress the importance of chilling, and often-quoted work is that of Schade (1926), who found that German soldiers exposed to wet and cold winds developed 4 times as many colds as those not so exposed

eating food prepared by a cook who was suffering from an acute cold Experiments of Hare and Mackenzie (1946) suggest that particles of secretion largely fall downward on to clothing; an important source of infection would be particles liberated later by shaking the clothing.

BACTERIOLOGY OF THE COMMON COLD

Needless to say, great interest has been taken in the bacteriological findings in cases of the common cold, and we can only refer to certain of the more important results (see Bloomfield, 1921 *a*, *b*.; Cooper, Mishulow, and Blanc, 1921; Valentine and Mishulow, 1921; Williams, Nevin, and Gurley, 1921; Jordan, Norton, and Sharp, 1923, Robertson and Groves, 1924, Shibley, Hanger, and Dochez, 1926; Blackburn *et al*, 1930, Hoyle, 1932, 1934, Thomson and Thomson, 1932).

1. *Examination in the acute stage.* Many workers have failed to find any specific organism, either in the nose or throat. The usual findings have been only the common throat organisms streptococci, staphylococci, pneumococci, influenza bacilli, *Micrococcus catarrhalis*, and so forth Generally there has been no predominant organism, and it has been impossible to incriminate any one more than another. During the early stages of colds, there may be a diminution in bacterial content as compared with the state of affairs either in health or during the later stages of the illness. In the purulent stage, bacteria are usually increased in number.

However, a number of authors has regarded bacteria as the primary cause of colds (see in particular Schroder and Cooper, 1930, Thomson and Thomson, 1932; Hoyle, 1932, 1934). These workers usually examined selected persons at frequent intervals During health probably only small numbers of pathogens were isolated, but on repetition during an attack of coryza profuse growths of one or more pathogens were obtained, and to these was attributed an etiological rôle. The organisms particularly incriminated by these workers were influenza bacilli and pneumococci Probably streptococci, *M catarrhalis*, and other organisms may also be responsible

With regard to anaerobic bacteria, it was at one time thought that these might be of etiological importance in colds However, as they have been found to occur frequently in the perfectly normal upper respiratory tract (Mills, Shibley, and Dochez, 1928), and in various other diseases (Burky and Freese, 1931), it is unlikely that their presence is of any consequence (see also Noble and Brainard, 1932).

2. *The bacteriology of colds in children.* In infants Kneeland (1930) could find no evidence that bacteria were responsible for first colds In recurrent colds of

induce an attack of cold

They noted that colds tended to be frequent in the autumn, these colds were of mild nature and unassociated with any change in the bacterial flora At this time of year there was a low carrier rate of pathogens, but after the outbreak this rose In mid-winter, when carriage of pathogenic organisms had become very frequent, a severe type of cold prevailed, and was of a different nature from the mild autumnal type These colds affected particularly children from 8 to 14 months old

These studies led the authors to suggest that the virus of the common cold *per se* caused the earlier, milder attacks. The effect of the virus was to increase the susceptibility of the tissues to pathogenic bacteria, and the severer and later colds were due mainly to the presence of these.

3 *The bacteriology of colds in isolated communities.* Burky and Smillie (1929) studied the nasopharyngeal flora in Labrador, and in southern Alabama, obviously places with two very different climates. In Alabama they recorded an increase in

or may occur in a zone next to the surface. The necrosis may extend deeply. By the third day, almost all the columnar cells have disappeared, and some of the stellate cells close to the basement membrane also slough.

After the acute stage, the remaining cells proliferate and form a syncytium. Eventually the cells lengthen and again appear columnar.

The secretion in the first few hours is watery, and contains comparatively few cells. Sometimes within the first 24 hours living ciliated cells appear. They are more numerous during the 2nd day, and disappear after the 3rd, when the secretion is purulent. They may disappear in a matter of a few hours. The deeper epithelial cells, polyblasts, and polymorphs appear on the 2nd day and become rapidly more numerous. The polymorphs are the predominant cells after a few days.

EPIDEMIOLOGY

Colds occurring in the tropics are said to be milder than those of more temperate lands. Studying Eskimos, Heinbecker and Irvine-Jones (1928) found that colds broke out within 72 hours of the arrival of an exploring party. The infection spread very rapidly to involve almost the whole village. After recovery, colds were not again experienced until a further visit of the explorers. Paul and Freese (1933) on Spitzbergen found that colds broke out on the arrival of the first boat of the year and rapidly spread through the population. Similar observations have been made in an Eskimo community (Høygaard, 1939), and on a Pacific island (Norris, 1944).

The Spread of Colds

By cases. Cases are the main factors in the dissemination of colds, and the filtrable virus has been recovered from the nasal and throat secretions, as have the various pathogenic organisms suspected of bearing a causal relationship to the condition. It is thus certain that the means of spread is by infected droplets. Colds are probably first infective late in the incubation period and for a day or so of the coryzal stage. Thus it has been recorded that two chimpanzees caught colds from a worker who, although appearing well at the time, developed a cold on the following day (Long, Bliss, and Carpenter, 1932).

It is a matter of common knowledge that colds are highly contagious, but exactly what mechanisms are responsible for their spread under natural conditions is hard to determine. For instance, Kerr and Lagen (1933–4, 1934) exposed a number of presumably susceptible volunteers to a person suffering from an acute cold. The volunteers and the case were segregated in a special isolated room, and there was every facility for contact infection. However, none of the volunteers contracted the infection. Thomson and Glazebrook (1941) found that an epidemic failed to develop in a community of naval trainees although conditions were very favorable to spread, and tonsillitis was in fact epidemic.

By carriers. There are few experimental observations dealing with the question of carriers of the causal agent. In temperate lands it would appear probable that such carriage does occur, for the following reasons. Colds frequently develop after exposure to cold, wet, or other adverse influence, without any known contact with a case of the disease. This suggests that the causal agent has lain latent in the nose or throat of the victim and has been awakened into activity. Moreover, even without exposure to adverse influences, colds frequently develop in the absence of any known exposure to a case of the disease, this can be explained by postulating either autogenous infection, or infection from an outside apparently healthy source. Thirdly, and less speculatively, it has been recorded that after the arrival of healthy explorers, Eskimos have developed colds, also, on Spitzbergen colds break out on the arrival of the first boat of the shipping season (*vide supra*). These observations strongly suggest the existence of carriers of the causal agent.

By fomites. It is probable that colds may be spread by fomites. Thus, Bliss and Long (1933–4) found that an otherwise isolated chimpanzee developed a cold after

There is thus abundant evidence that the causal agent of some colds is filtrable. We shall first describe the characters of the virus isolated by Dochez. We are not, as yet, prepared to accept this agent as the cause of all colds, although it is probably responsible for some.

Properties of the Common Cold Virus (Dochez)

Infection in chimpanzees.

Dochez and his coworkers have shown that the ape can be readily infected with material from human colds (see, e g, Dochez, Shibley, and Mills, 1928–9, 1930, Shibley, Mills, and Dochez, 1930, see also below).

1 *Natural infection* Natural infection with colds may arise quite readily, thus, attendants in zoological gardens report that colds are quite common among apes In experimental laboratories also, these animals are very readily infected from human beings, unless rigorous quarantine is carried out.

2. *Experimental infection.* Infection is readily initiated by instilling about 1 c.c. of filtered nasal secretion from human cases of common cold. The disease develops after about 36 to 48 hours' incubation period. The animals are sluggish, and the eyes appear puffy; there is a mucoid secretion in, and obstruction of, the nasal passages. The animal coughs and sneezes, and after a few days the nasal secretion becomes mucopurulent.

3. *Control observations.* Inoculation of chimpanzees with broth and other fluids has never produced such manifestations of infection, further, Shibley, Mills, and Dochez (1929–30) have shown that intranasal inoculation with filtered nasal washings of normal persons has no effect.

4 *The rôle of bacteria in experimental infection.* It has been reported that the nasopharyngeal flora of chimpanzees in health is very similar to that of man (Dochez, Shibley, and Mills, 1930). However, with the onset of experimentally induced colds the same authors found a change to occur in the flora, Pfeiffer's bacillus was easily recognized, and from throat cultures pneumococci could be isolated.

Further, Dochez, Mills, and Kneeland (1932–3) found that an interesting change might occur after chimpanzees had been inoculated intranasally with their cold virus Strains of *B influenzae* which, in health, were rough and avirulent now changed into the "S" or virulent form

Infection in other animals.

Edwards (1935–6) reported that hedgehogs might develop catarrh after nasal inoculation of infective human filtrates. It appears that very trivial lesions may be produced in mice (Chapman and Hyde, 1940, Powell, Sparks, and Clowes, 1940).

Filtrability and cultivation.

The agent has been found to pass all grades of Berkefeld filter (see, e g, Kruse, 1914, Foster, 1916, Shibley, Mills, and Dochez, 1930, Dochez, Shibley, and Mills, 1930, Long *et al*, 1931). It also passes a Seitz filter (Dochez, Mills, and Kneeland, 1930–1, Long *et al*, 1931)

It has been shown that the virus of the common cold can be cultivated *in vitro* (see Dochez, Mills, and Kneeland, 1930–1, 1931, 1931–2, 1936, Powell and Clowes, 1931–2, Powell, Sparks and Clowes, 1940).

The virus is grown in chick embryo and buffered bouillon or Tyrode's solution, according to the technique elaborated first by the Maitlands and later by Li and Rivers (see Ch XII). The cultures should be transferred at 2- to 3-day intervals to obtain the best results.

Virus grown in tissue culture is still able to produce colds in human volunteers on suitable inoculation. Thus, Powell and Clowes found that the 27th generation

the number of Pfeiffer's bacilli and pneumococci in association with colds. The bacterial flora [illegible] Indies, was ex[illegible] much less frec[illegible] found that th[illegible] and Alabama They found no change in the flora in those who developed colds The nasopharyngeal flora of dwellers on Spitzbergen was examined by Paul and Freese (1933). They did not find any evidence that the bacterial flora was concerned in the causation of colds With the possible exception of Burky and Smillie's work, it is thus evident that these studies have done nothing towards establishing bacteria as the cause of the common cold.

4 *Bacterial vaccines in the prevention of colds.* Much has been written on the subject of the prophylaxis of colds by vaccination. Vaccines, either stock or autogenous, have usually contained staphylococci, streptococci, *Micrococcus catarrhalis*, Pfeiffer's bacilli, and other organisms.

There is no doubt that many have expressed satisfaction with their results (see e.g., Thomson and Thomson, Veasey, 1941; Walsh, 1940, 1941; Herron, 1943). Such vaccines may be injected subcutaneously, intradermally, orally, or nasally. There has, however, been a number of investigations, some on a large scale, which entirely failed to show that vaccines have any prophylactic effect (von Sholly and Park, 1921, Ferguson, Davey, and Topley, 1927, Wallfield, 1937, Diehl *et al*, 1938, Siegel *et al*, 1943, Council on Pharmacy and Chemistry, 1944; Cowan and Diehl, 1944, McGee *et al.*, 1944, Bourne, 1946).

THE VIRUS THEORY OF THE COMMON COLD

Early Experimental Observations

Negative transmission experiments. Certain authors have failed in their efforts to transmit the infection by filtrates of nasal or nasopharyngeal secretions (e.g., Williams, Nevin, and Gurley, 1921, Robertson and Groves, 1924). The inoculations were given intranasally to human volunteers

Kerr and Lagen (1933–4, 1934) found that intraconjunctival inoculation of infected nasal secretion failed to transmit the disease. Further, they failed to transmit the disease by placing a case in an isolated room with susceptibles. Despite these negative results, numerous authors have succeeded in transmitting the infection

Positive transmission experiments. Kruse (1914) was probably the first clearly to suggest the possibility of a filtrable virus being the cause of colds He produced infection in human volunteers by inoculation with filtered nasal secretion of coryzal cases Later Foster (1916, 1917) prepared Berkefeld filtrates from the nasal secretion of cases of colds, and 90 per cent of volunteers injected with these filtrates developed symptoms within 8 to 30 hours Dold (1917) also obtained positive inoculation results with filtrates Olitsky and McCartney (1923) found that the filtered throat washings of cases of colds could transmit the infection to human volunteers By means of Berkefeld filtrates of the nasal secretion of cases, Dochez and his colleagues were able to infect human volunteers (Shibley, Mills, and Dochez, 1930, Dochez, Shibley, and Mills, 1930) Andrewes and Oakley (1931–2) are also reported to have infected human volunteers

Human inoculation experiments were likewise successfully carried out by Long, using as inocula filtrates of secretion (Long and Doull, 1930–1, Long *et al*, 1931) The infection was passed through a number of persons in series Walker (1931–2) has pointed out that care is needed in interpreting the results of inoculation tests, volunteers may imagine that they have actually developed a definite cold when they have been inoculated intranasally only with control noninfective material

More recently positive results have been obtained by Andrewes and his group, and by the Commission on Acute Respiratory Diseases (see below)

ogous SCC filtrate developed illnesses, although these were somewhat milder than on the first occasion.

9. No cross resistance could be demonstrated between the causal agents of ARD and the common cold.

10. In further experiments, it was confirmed that primary atypical pneumonia could be induced by means of bacteria-free filtrates Previous infection with ARD and SCC filtrates did not give any resistance to AP

11. To summarize the work reported in these two papers, it appears that at least 2 filtrable agents may induce minor respiratory illnesses in man, and these viruses are probably distinct from the causal agent of primary atypical pneumonia Pollard and coworkers have reproduced colds with Seitz filtrates of nasal washings stored at −20° C for up to 115 days (Pollard, Dernehl, and Caplovitz, 1948).

IMMUNITY TO THE COMMON COLD

Chimpanzees recovered from natural or experimental cold infection are resistant to reinfection for 3 to 4 months (Dochez, Shibley, and Mills, 1930). There is dubiety as to whether immunity follows infection in man, but probably there is a transient resistant state. Thus the observations of Paul and Freese (1933) at Longyear City, Spitzbergen, showed that immunity followed an attack and lasted for 3 to 7 weeks Investigating the disease in a community of naval trainees, Thomson and Glazebrook (1941) could find no evidence of immunity resulting from overt or subclinical attack.

PREVENTION AND SPECIFIC TREATMENT

In institutions, the incidence of colds can be lowered by spraying with propylene glycol (Harris and Stokes, 1943). A number of reports has alluded to the lowered incidence of respiratory infections, including presumably colds, by measures directed to lessening dust, ultraviolet irradiation is also effective. It has been claimed that the incidence can be lowered by periodical injections of gamma globulin (Adams and Smith, 1946). There is no evidence that the administration of vitamins lowers incidence (Cowan, Diehl, and Baker, 1942; Dahlberg, Engel, and Rydin, 1942)

Preliminary reports on the value of patulin in treatment (Raistrick *et al*, 1943) have not been confirmed (Patulin Clinical Trials Committee, 1944; Stansfeld, Francis, and Stuart-Harris, 1944) Sulfonamides do not shorten the course of the disease, but control secondary invaders, and lessen the incidence of complications (Cecil, Plummer, and Smillie, 1944, Faust and Simmons, 1944).

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produced severe colds in 75 per cent. of inoculated persons Andrewes and Oakley (1931–2) are reported to have failed to infect London volunteers with Dochez's cultures sent from America. Dochez, Mills, and Kneeland (1938), in a preliminary series of experiments, found that immunization of human beings with tissue culture virus was ineffective.

Only trivial lesions result from chorio-allantoic inoculation (Kneeland, Mills, and Dochez, *1936–7*; Hyde and Chapman, 1937; Peragallo, 1938, Chapman and Hyde, 1940)

Preservation.

To preserve the virus for some length of time various procedures may be adopted (Dochez, Mills, and Kneeland, 1930–1, 1936):

(*a*) Nasopharyngeal washings may be frozen and desiccated *in vacuo*, when virulence is preserved for at least 4 months

(*b*) Nasopharyngeal washings, kept anaerobically in the refrigerator (with cysteine added), remain virulent for at least 13 days.

(*c*) *If gum acacia is mixed with infected tissue culture, then frozen and dried in vacuo*, virulence is preserved

Later Experimental Observations

The British Medical Research Council is sponsoring experiments conducted by Dr. C. H. Andrewes on human volunteers, and this group has shown that there is no doubt that mild colds can be transferred by means of filtrates (see Andrewes *et al.*, 1947, *Brit. med J*, 1947, *1*, 650) The agent can be stored for some months at −76° C. (Fulton, 1947).

Some evidence pointing to growth in the allantoic cavity has been obtained (Pollard and Caplovitz, 1947, 1948, Topping and Atlas, 1947)

Experiments have been carried out by the U.S. Commission on Acute Respiratory Diseases (1947 *a*, *b*, Dingle, 1947).

1. As a control, a number of volunteers were first inoculated with filtrates prepared from their own nasopharyngeal washings No illness was produced

2. Of 14 volunteers inoculated with filtrate from a case of ARD (see above), 12 developed minor respiratory illness, and 2 remained well. Symptoms were first *observed in 9 persons on the 5th–6th day after inoculation*

3. Next, 14 volunteers were inoculated with filtrate from a case of SCC, and 9 developed illness within 24–48 hours. The features were those of "common cold," and could be distinguished from those following the ARD inoculation

4 Some 10 volunteers were given filtrate from a case of common cold Six developed common colds. The onset of the illness occurred within 4 days of inoculation

5. A group of 10 volunteers was given filtrate from a case of Br-AP, 4 developed minor respiratory illnesses, without fever None of the illnesses was as severe as that in the donor of the inoculum

6. There were no consistent alterations in bacterial flora, serological tests, or tests for influenza virus in these groups

7. Discussing these results, the Commission point out that only 2 types of ill- [illegible] -s of the various inocula The first type, [illegible] by pharyngeal involvement, and an in- [illegible] type, induced by CC and SCC filtrates, was characterized chiefly by coryza, and an incubation of 24–48 *hours* *The* evidence suggested that the causal agent of the common cold is a virus, ARD is probably caused by a distinct virus

8 None of 6 individuals receiving ARD filtrate 3 weeks after a previous inoculation developed evidence of respiratory illness, 4 of 5 men inoculated with homol-

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Virus was recovered from the conjunctival secretion by allantoic inoculation There were no general symptoms, and there was no increase in antibody. In the second case, a laboratory assistant complained of slight conjunctival irritation, with a mild regional adenitis. Virus was recovered from the tears. Workers in Palestine have also described conjunctivitis due to laboratory infection, as well as small outbreaks in persons preparing infected poultry for the table (Shimkin, 1946; Yatom, 1946).

Some Properties of the Virus

The virus was found to measure 80–120 mμ as determined by ultrafiltration, whereas that of fowl plague measure 60–90 mμ (Burnet and Ferry, 1934).

Purified preparations examined in electron micrographs may show sperm-like forms (Bang, 1946, Cunha *et al*, 1947). However, if, after centrifugation, the deposit is suspended in water instead of saline, the virus shows a preponderance of spherical forms; on adding saline, the filamentous form is assumed, this change can be prevented by partial inactivation with formaldehyde, mustard gas, or gentle heat (Bang, 1947)

The infection can be transmitted to poultry and pigeons by inoculation of filtrates prepared from the organs of dead birds (Doyle, 1927).

Mice may show a consolidation of the lungs when inoculated intranasally on a

tion of virus inter-

The virus can be

The virus grows in the egg, and can be inoculated by the amniotic and allantoic routes, and on the chorio-allantois (Burnet and Ferry, 1934; Iyer and Dobson, 1940, Burnet, 1942; Burnet, 1943, Lush, 1943, Anderson, 1946, Beveridge and Burnet, 1946, Burnet and Anderson, 1946, Cunha *et al.*, 1947).

Foci are developed on the chorio-allantois, especially with more dilute preparations, it becomes abnormally moist and edematous. Small petechial hemorrhages are seen. There is patchy ectodermal proliferation, followed by vacuolation and necrosis (Burnet and Ferry, 1934). The embryo dies from hemorrhagic encephalitis, hemorrhages are also found in the pectoral muscles, skin, and intestines (Burnet, 1942, 1943). On chorio-allantoic passage, the virus becomes less virulent for fowls (Iyer and Dobson, 1940).

After amniotic in

is turbid, and shows

found in the embry

Virus grows well in the allantoic fluid on intra-allantoic inoculation (Burnet, 1942).

A fatal infection with hemorrhagic lesions follows intravenous inoculation (Beveridge and Burnet, 1946).

The virus can be titrated by inoculation on the chorio-allantois, in the amniotic or allantoic cavity, or by chick cell agglutination *in vitro*. The intravenous route can also be used (Hanson, Winslow and Brandly, 1947)

The virus is resistant to glycerin (Burnet and Ferry, 1934). The maximal stability occurs between pH 4 and 11 (Moses, Brandly, and Jones, 1947).

Cunha *et al* (1947) have purified the virus by centrifugation, and found that it is a complex of protein (approx 67 per cent.), lipid (about 27 per cent.), and a small amount of nucleic acid, some of which is of the desoxypentose type

Hemagglutination Produced by NDV

Newcastle virus causes agglutination of chick and other red cells (see Ch LXI), as has been shown by Burnet, his collaborators, and others (Burnet, 1942; Clark

CHAPTER LIII

NEWCASTLE DISEASE[1]

Original Description

DOYLE (1927) first described the features of a virulent infection of poultry that occurred in 1926. The first outbreak was near Newcastle-on-Tyne, and other districts were involved later. The incubation period was 4–11 days The disease resembled, but could be differentiated from, fowl plague The mortality was about 100 per cent. and the disease was evidently spread by contact.

A filtrable virus was isolated from the body fluids, organs, and excretions.

Geographical Distribution

Outbreaks of Newcastle disease, due to immunologically identical strains have occurred in many parts of the world, for example Dutch East Indies, Philippines, India, Ceylon, Korea, Japan, Kenya, Palestine, Italy, Germany, and the U. S. A (Doyle, 1935, Beaudette, 1946; Brandly *et al*, 1946).

The disease occurred in a poultry flock in Victoria in 1930, and became widespread in Melbourne, it reappeared in 1932 (Albiston and Gorrie, 1942). Fowls were chiefly affected, but also turkeys. Ducks and geese showed a marked resistance. The death rate of the natural disease varied from 50–100 per cent The fowls showed dullness, somnolence, mouths sticky with exudate, diarrhea, and final prostration and twitchings, some cases of chronic infection were seen Postmortem examination showed generalized hemorrhages, ecchymoses in the proventriculus, and intestinal submucous hemorrhages. The infection could be transmitted artificially. Tests performed by Doyle showed that the agent was a strain of Newcastle disease virus (NDV).

The condition of avian pneumo-encephalitis of young chickens first found in California but now elsewhere in the U S A, and the disease of mature birds known as "chicken flu," is caused by a filtrable virus immunologically identical with that of NDV (Beach, 1944, 1946).

The methods of laboratory diagnosis in suspected outbreaks are discussed by Beaudette (1946) and Osteen and Anderson (1948).

During World War II the disease was intensively studied by workers in the U S. A and much important information obtained (Beach, 1946, Brandly *et al*, 1946).

Human Infection

Burnet (1943) described an infection due to squirting of infectious allantoic fluid into the eye The next morning there was an acute conjunctivitis, and the right preauricular gland was swollen and tender There was a headache, slight feeling of chilliness, and discomfort The following day there was an improvement The inflammation subsided in 2 weeks

The virus was recovered by amniotic inoculation Antibody could be detected in the convalescent serum by a chick-cell agglutination test, and by the inoculation of serum-virus mixtures on the chorio-allantois

Two further laboratory infections have been described by Anderson (1946) In one case, a mild bilateral conjunctivitis followed the splashing of virus in the face

[1] Newcastle disease primarily affects birds, man is only rarely infected Consequently, we shall discuss the properties of the virus only briefly, and with particular reference to its biological relationship to influenza virus

may agglutinate NDV-treated cells, and that this phenomenon is not specific (Evans and Curnen, 1948).

Action of anti-Rh sera.

When human cells were treated with NDV, only the Rh positive cells, containing D Rh antigen, were agglutinated by the incomplete anti -D Rh antibody (Chu and Coombes, 1947).

IMMUNITY

It has proved possible to immunize poultry with vaccines produced in the egg (Beach, 1946). Hamster-adapted virus has also been used (Reagan *et al*, 1948).

Antisera can be produced in rabbits and ferrets by injections of the virus (Burnet, 1942). Antibodies develop in the sera of fowls injected with formolized virus, followed by live virus, and in the sera of guinea-pigs given intravenous or intracardiac injections (Burnet and Anderson, 1946). Antibody is found in the sera of human convalescents (Burnet, 1943).

Antibody can be titrated by CCA-inhibition tests (Lush, 1943; Burnet and Anderson, 1946, Florman, 1947, or by cells with serum (Burnet and Anderson, the view that the antibody titrated by cells treated with NDV are agglutinated to a lower titer by antisera to swine virus.

Antigenic Relationship to Other Viruses

Influenza Despite their similar biological properties, serological tests show no antigenic relationship between the two agents (Burnet, 1942) The morphology shown by the electron microscope (see above) is certainly different from that of influenza virus It seems difficult to believe, however, that the similarities shown by the hemagglutination reaction do not indicate a close relationship between the two agents

Mumps. As has been explained, the hemagglutination reaction shows that the two viruses have similar properties, and fall in the same linear series Cells treated viruses Cells treated with mumps a reduced titer (Burnet, 1945) hought that the disease is only a ιle (1927) differentiated the two agents by cross immunity tests, and found that pigeons could be infected with Newcastle, but not fowl plague virus The two agents can be differentiated by many biological properties, including size, and the appearance of infected eggs (Burnet and Ferry, 1934). Lush (1943) using CCA-inhibition tests, found no relationship between the two agents

Other viruses Beach (1944) has shown that the viruses of avian pneumo-encephalitis of young chickens and "chicken flu" are apparently immunologically identical with NDV.

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1. Agglutination is best recognized by studying the pattern of the cell sediment, rather than by clearing of the supernatant.

2. Human cells are not quite so readily agglutinated as fowl cells, but otherwise behave similarly.

3. Heating and formalin render NDV noninfective, but hemagglutinating capacity is retained.

4. Seitz filtration causes marked loss of infectivity, while the hemagglutinating titer is hardly lowered, suggesting that particles smaller than the virus are responsible for some of the hemagglutination. Cunha *et al.* (1947), however, found that infectivity and hemagglutinative activity were correlated with the presence of virus particles.

5. Agglutinated cells rapidly elute virus, and thereafter become completely inagglutinable or "stabilized." Thus, when tubes containing fowl cells agglutinated by NDV at room temperature are shaken, the cells usually fail to reagglutinate When such cells are washed and resuspended, they form a stable suspension Elution and stabilization can also be shown by adding human cells to serial dilutions of virus, and holding at 37° C Deposition is almost complete at 1 hour If the rack is then shaken and allowed to stand, by the end of the 2nd hour, all tubes containing over about 40 agglutinating doses show stabilized inagglutinable cells. At 4° C, elution does not take place so quickly.

6. Cells treated with ND virus in this way, and made up in the form of a stabilized suspension, can no longer be agglutinated by the homologous virus or by mumps virus, but are agglutinable by influenza viruses. This is because mumps, NDV, and influenza viruses can be arranged in a linear series, such that when red cells are treated with virus, and then exposed to the action of other viruses, the cells are not agglutinated by the virus used to treat them, or by viruses preceding this one in the series, but are agglutinated by viruses later in the series (see Ch. LXI).

7 Florman (1947) recommends the performance of hemagglutination tests at 4° C. instead of room temperature.

Agglutination of NDV-treated cells by sera.

1 Cells treated with NDV can be agglutinated by specific antisera prepared in animals, and this can be used as a means of titration of antibody.

2. Nearly all normal human sera agglutinate human red cells treated with NDV, to a titer *below 1, 120 This phenomenon is probably related to Thomsen's panag*glutination of red cells by bacteria (Burnet and Anderson, 1946, Chu and Coombes, 1947).

3. The majority of sera from cases of infectious mononucleosis agglutinates NDV-treated cells to a titer over 1, 120, fowl, guinea-pig, and human cells can be used (Burnet and Anderson, 1946)

4. The sensitizing factor in NDV which renders red cells susceptible to agglutination by sera from cases of infectious mononucleosis is probably not ND virus itself. The factor is probably adsorbed to the surface of the red cell

5. There are 3 possible explanations for this agglutination of NDV-treated cells by mononucleosis sera (*a*) The etiological agent of infectious mononucleosis is related antigenically to NDV (*b*) The relationship is accidental, and analogous to that between *Rickettsiae* and *Proteus* (*c*) A labile globulin is developed in the serum in infectious mononucleosis, and this by some accident of molecular structure reacts with NDV as well as sheep red cells (Burnet and Anderson, 1946)

6. Later work suggests that the sera of patients suffering from several infections

CHAPTER LIV

PANDEMIC INFLUENZA (1918–19)

Introduction

INFLUENZA is a disease which has been known for several centuries, although it is not always certain that the diagnosis in some of the earlier outbreaks is beyond suspicion. There was no unanimity regarding the causal agent until recently, when, in 1933, British workers reported the isolation of a filtrable virus This agent has now been isolated in many parts of the world and well-nigh universally accepted as the etiological agent. Prior to 1933 many believed Pfeiffer's bacillus to be the causal agent, it is now usually believed, however, that its rôle is secondary rather than primary.

The world has been visited by a number of comparatively well-authenticated pandemics of influenza since about the beginning of the 12th century In comparatively modern times such pandemics occurred in 1847–8, 1889–91, and 1918–19. Those who are interested in the history of influenza should consult the works of Hirsch (1883),[1] Creighton (1891, 1894), and Townsend (1933). This chapter will be concerned almost entirely with describing the 1918–19 pandemic, which actually resembled the 1889–91 outbreak closely. This 1918–19 outbreak was experienced in all parts of the world The disease had a high attack rate, pneumonic complications were common, and these cases had a high mortality rate The disease occurred in 3 main waves, spring to summer 1918, autumn 1918, and spring 1919. Influenza has not occurred in pandemic form since this period

Much literature has been published on influenza, in particular from 1918 to 1920, and since 1933. A number of excellent monographs has appeared, which should be consulted by those who desire further information on, and references to, work carried out prior to the isolation of the virus in 1933 (e g, *Medical Supplement*, 1918, Ministry of Health, 1920, Crookshank, 1922, Jordan, 1927, Scott, 1929, Thomson and Thomson, 1933, 1934, Burnet and Clark, 1942). The work of the Thomsons is the most outstanding and complete, as over 4,500 papers are reviewed

CLINICAL FEATURES OF PANDEMIC INFLUENZA

Premonitory illnesses In 1915 influenza was prevalent in the United States, in Great Britain, and in later years in other countries, but these outbreaks did not spread widely During 1917, British authors drew attention to the frequent occurrence of an unusual condition which appeared to be almost a "new disease"—purulent bronchitis (Hammond, Rolland, and Shore, 1917, Abrahams *et al.*, 1917, see also 1919) This condition occurred particularly in troops in the Aldershot Command (England) and in France, and was characterized by an abundance of mucopurulent to frankly purulent sputum, and cyanosis. *B influenzae* was isolated in many cases, but usually associated with other throat organisms. The disease did not show any tendency to spread among the populace, but it was of interest as it had many features of true influenza

Incubation period Countless observations proved this period to be 24 to 48 hours.

Onset The onset of the disease was characteristically sudden There might be a single case in a community on a given day, every one else feeling in perfect health, then, on the next day thousands might be affected Many a person was hit-

[1] References are appended at the conclusion of Ch LXIII, p. 671 et seq

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might be a degree of absolute lymphocytosis. Leukocytosis might be observed in cases complicated by pneumonic conditions

BACTERIOLOGY OF PANDEMIC INFLUENZA

Needless to say, influenza attracted the widest interest, and countless workers reported the results of their bacteriological investigations (e g, in *Great Britain*: Influenza Committee, 1918, Fildes, Baker, and Thompson, 1918, Hopkins, 1918–19, McIntosh, 1918, 1922, Whittingham and Sims, 1918, Logan, 1919, 1921, Fildes and McIntosh, 1920, Scott, 1922; Donaldson, 1922, in *France*: Influenza Committee, 1918, Tytler, Janes, and Dobbin, 1919, Gibson and Bowman, 1919, in the *United States*: Blanton and Irons, 1918, Camp Lewis Pneumonia Unit, 1919, Park, 1919, Pritchett and Stillman, 1919, Stone and Swift, 1919, Williams, 1919, Opie *et al.*, 1919 *b*, in *Germany*: Bernhardt, 1918, Dietrich, 1918, Simmonds, 1918, in *India*: Greig, 1920–1) Those who desire fuller information should consult Thomson and Thomson (1933, 1934) or Jordan (1927).

Specimens were usually taken from throat swabs, garglings, or sputa. *B. influenzae* * was frequently found in such specimens, either in pure culture or more frequently along with the usual throat flora In fact, certain observers found the organism in over 80 per cent. of cases A fuller discussion of the rôle of *B influenzae* is found below

A variety of organisms was isolated by postmortem lung culture The severest lesions were produced by *B. influenzae*, *Pneumococcus*, hemolytic streptococcus, and *Staphylococcus aureus* In certain cases nonhemolytic streptococci were isolated (e g, by Blanton and Irons, 1918, Gibson and Bowman, 1919) *Neisseria* organisms were also isolated, some at least being true meningococci (Fletcher, 1919).

With regard to serological observations, it was reported that the sera of influenza convalescents fixed complement with influenza bacilli as antigen, and that agglutination also occurred (e g, Sobernheim and Novakovic, 1918, Gibson and Bowman, 1919, Gay and Harris, 1919, Rapoport, 1919, Scheer, 1919, Cooke, 1920, McIntosh, 1922, Sharp and Jordan, 1924) The agglutination was probably not specific, as serum of measles convalescents was also found to agglutinate *B influenzae* (Sharp and Jordan).

MORTALITY

Although the total number of deaths throughout the world was enormous, the average mortality rate was not much over 3 per cent. Death was invariably produced by some respiratory complication French (1920) found that about 20 per cent of cases developed pneumonia in the 2 later waves, and of these nearly half died In the United States of America it was estimated that 550,000 deaths occurred, and in Great Britain 220,000 In the whole world the total death rate probably amounted to over 20 million (Jordan, 1927) The majority of deaths occurred in persons between 15 and 45, especially in the later waves This feature was unusual as previously influenza, even in pandemic form, had tended to show maximum mortality figures for those over 60 years of age The tendency to cause a fatal result in young adults was one of the main characteristics of pandemic influenza in 1918–19 The pandemic increased the total death rates, deaths from respiratory diseases, and deaths from diseases of various other systems, such as the heart.

PATHOLOGY

The Respiratory System

Virtually every fatal case of influenza in 1918–19 presented evidences of pulmonary involvement, the other organs of the body being only slightly affected.

* In this chapter we retain the old name, *B influenzae*, rather than *Haemophilus influenzae* which has come into general use only since the days of pandemic influenza.

erally rendered acutely ill within an hour or so of first feeling unwell. Rigors were often experienced at the onset, headache and *fever soon developed*, while *epistaxis* was a common finding. In another type of case the disease was ushered in with intense congestion of the nose, pharynx, larynx, trachea, and conjunctivae (see Bloomfield and Harrop, 1919). In certain cases the illness developed as a sudden increase in severity of a preexistent coryza, headache, or cough.

Course. There was considerable difference between the course of the cases seen in the summer of 1918 and those seen in the 2 later waves, pneumonia developed in about 20 per cent. of the later cases but was very rare in the earlier. We will record the clinical features as described by French (1920), who saw some thousands of British cases during the 3 waves, and was able to correlate his clinical and pathological findings.

Cases in the first wave This type of illness was often known as 3-day influenza The onset was rapid, the patient being seized with intense lassitude, the temperature remained raised for about 2 days to fall by the third There was intense headache and general aching, the face was flushed, the tongue furred, the throat sore and red, and the voice husky. The patient was usually well within a week, and complications were rare.

Cases in the later waves. French estimated that about 80 per cent. of the cases seen in autumn 1918 and spring 1919 were comparatively mild, were devoid of complications, and resembled those seen earlier. The remaining 20 per cent, however, suffered from pneumonia and nearly half died

Pneumonic cases. The pneumonia of influenza might develop in various stages of the illness (French)

(*a*) After a day or so of mild illness the patient rapidly became worse and showed evidence of lung involvement.

(*b*) Some cases were stricken with pneumonia from the onset.

(*c*) Pneumonia developed late in some patients, after about a week of comparatively mild influenza.

(*d*) In some cases the pneumonic process developed later, during convalescence

Pain in the chest was commonly complained of, usually either behind the sternum, basally, or just below the nipple. The respiration rate was greatly accelerated, even up to 60 per minute The pulse, however, was usually powerful The cough was short and hacking initially, but later was softer, when sputum was being expectorated Cyanosis was observed in many of these pneumonic cases.

Cyanosis A characteristic feature of the disease was a heliotrope cyanosis, involving the face and chest and sometimes other parts of the body (for illustrations see Abrahams, Hallows, and French, 1919, French, 1920) French estimated that approximately half the cases of pneumonia had flushed red faces while the remainder were cyanosed He estimated the mortality rate of cyanotic cases at 95 per cent, the patient might die in 24 hours or linger for as long as a week Another characteristic appearance was a vermilion color of the pharynx and soft palate (see Bloomfield and Harrop, 1919)

Other complications No attempt will be made to discuss complications other than pneumonia. The following list of possible complications is that given by French. epistaxis, hemoptysis, and hematemesis, subsultus and delirium, rupture of the rectus muscle, superficial thoracic emphysema, sinusitis, parotitis, otitis media, and meningitis, jaundice and nephritis, herpes.

Sequelae Even in comparatively mild cases return to health was slow. There was intense physical and mental weakness, the patients being usually unfit for any exertion, and depression was common.

The blood picture Numbers of authors studied the blood picture in influenza (e.g, Bloomfield and Harrop, 1919, Chickering and Park, 1919, Camp Lewis Pneumonia Unit, 1919, Bunting, 1921) It is probable that in the early stages there was a neutrophilia which was shortly followed by the characteristic leukopenia, there

might be a degree of absolute lymphocytosis. Leukocytosis might be observed in cases complicated by pneumonic conditions.

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The lesions varied from case to case and locality to locality. Many of these were quite novel and were studied by numerous authors, of whom only comparatively few can be mentioned by name (Fildes, Baker, and Thompson, 1918; Lubarsch, 1918, Oberndorfer, 1918; Simmonds, 1918, Hopkins, 1918–19, LeCount, 1919 *a, b, c*, Champtaloup and Drennan, 1919; Chickering and Park, 1919; Connor, 1919, Goodpasture, 1919 *a, b*; Goodpasture and Burnett, 1919, MacCallum, 1919, Opie *et al*, 1919 *b*; Stone and Swift, 1919; Sundell, 1919; Tytler, Janes, and Dobbin, 1919, Wolbach, 1919, French, 1920, McNamara, 1920, Symmers, Dinnerstein, and Frost, 1920). In addition, Opie (1928) has reviewed the writings of British and American authors, while Luksch (1928) has reviewed the German work. French (1920) appends to his article a full bibliography.

We shall describe the changes that might be encountered, first on an anatomical, and later on a bacteriological, basis

1. *Pleural inflammation.* Fibrinous exudates were commonly found overlying areas of consolidation, in other cases the pleural cavities contained varying quantities of blood-stained serous fluid. Empyemata were seen in certain cases, although sometimes it was difficult to find lesions in the lung; the causal organism in such cases was usually the hemolytic streptococcus.

LeCount (1919 *a*) drew attention to a lesion which he regarded as almost pathognomonic the subpleural area was distended with fluid and in parts the pleura was raised off the lung in characteristic plaques, through which some of the usual carbon mottling could be seen.

2. *Tracheitis and bronchitis* The trachea, bronchi, and bronchioles were usually acutely inflamed, and frequently contained purulent exudate. Bronchiectatic dilatation was observed in some cases of longer duration.

3. *Capillary necrosis* LeCount (1919 *b*) described disseminated necrosis of the interalveolar capillaries, best seen in small pneumonic areas. There was little cellular exudate associated with this necrosis of the interalveolar network. The necrosis was probably a purely toxic reaction and appears to have been responsible for the characteristic hemorrhages.

4 *Hemorrhages* The appearance of red hemorrhagic infarcts was one of the most characteristic features of the pathology, these were found subpleurally and in the lung parenchyma Microscopically, hemorrhage was frequently seen in the alveoli in areas where no gross hemorrhage might be visible naked-eye

5 *Interstitial emphysema.* This might follow necrosis of the alveolar and interalveolar tissue, when air tracked along the interstitial tissue to the hilum, and thence to the mediastinum

6 *Edema* The lungs were often bulky and edematous, and on section great quantities of hemorrhagic fluid escaped At the autopsy the bronchi and trachea were filled with fluid, which even ran out of the mouth and nostrils

7 *Abscesses* Abscesses were sometimes found in the lungs, especially in staphylococcal pneumonia, and might occur in the center of a patch of consolidation, subpleurally, or peribronchially

8 *Hyaline membrane* Certain authors recorded the presence of dilatation of the *ductuli alveolares* associated with a hyaline membrane covering the alveolar walls (Goodpasture, 1919 *a, b*, LeCount, 1919 *b*, MacCallum, 1919, Wolbach, 1919) The membrane was of irregular thickness and might completely fill an alveolus, being most conspicuous in acute pneumonia of short standing It did not give fibrin reactions, but was thought to be derived therefrom, along with necrotic cells, it was probably produced by the action of the virus *per se* and not by any particular type of secondary bacterial invader In 2 cases hyaline membrane was found in lungs that proved sterile on routine cultural examination, thus suggesting that the virus alone was responsible (Goodpasture)

Regarding the source of the alveolar fibrinous exudate, Wolbach has suggested that it is formed by the entrance of secretion from the bronchioles and alveolar

ducts at a time when air can enter to distend the alveoli Although formerly regarded as a specific feature of influenza, this is probably not now acceptable Thus Brannan and Goodpasture (1924) found a similar membrane in 2 cases definitely not influenzal, while Farber and Sweet (1931) found a similar membrane in the bronchioles and alveoli of newborn children, as a reaction to the presence of amniotic fluid The membrane was fatty and stained with scharlach R

9. *Fulminating influenza* In these cases death usually supervened a few hours after onset At *postmortem* the lungs were voluminous and on section poured out edematous fluid, subpleural and parenchymal hemorrhages were present

10. *Pneumonic consolidation* The majority of cases presented evidences of some consolidation, lobar or bronchopneumonic in distribution. Lobar pneumonia was more commonly reported in some areas than others, thus Opie *et al.* (1919 *b*), at an American army camp, found it in 50 per cent. of autopsies The more usual finding elsewhere, however, was bronchopneumonia, lobar pneumonia was only rarely found (e g, by French, 1920).

The characteristic bronchopneumonic nodules were reddish-gray in color and either discrete or confluent. The cut surface of the lung was usually hemorrhagic, and the bronchi contained purulent exudate. The pneumococcus, hemolytic streptococcus, and Pfeiffer's bacillus were frequently isolated from these cases (*vide infra*). In the earlier cases in the autumn of 1918 the bronchopneumonia was said to be more of a toxic injury than an inflammation, with hemorrhage and exudation of fibrin into the alveoli (see, e g, Goodpasture, 1919 *a*). In the early months of 1919, however, the same observer reported a more inflammatory type of reaction There were grayish-purple areas of lobular consolidation and much pus, while, microscopically, streptococci and pus cells were seen in the alveoli.

11. *Repair.* McNamara (1920) in particular has drawn attention to the reparative process that might be seen. Thus bronchiolar and alveolar exudates underwent organization with resultant fibrosis and bronchiectasis He also described an unusual, almost neoplastic-like, proliferation of the epithelium lining the bronchioles

An attempt was made to classify certain types of pneumonia according to the causal organism.

[illegible]

present, usually in the dependent parts of the organ These abscesses were clearly visible subpleurally and might be associated with pleural exudation. The lung usually showed, in addition, numerous hemorrhagic areas (see also Tytler, Janes, and Dobbin, 1919).

(*b*) *Pneumococcal pneumonia* A number of authors isolated pneumococci from the lung (e g, Blanton and Irons, 1918, Pritchett and Stillman, 1919, Logan, 1919, 1921, Opie *et al*, 1919 *b*) This condition was especially studied from the pathological point of view by MacCallum (1919). [illegible]
mal pleurae and bronchi, but, on section, [illegible]
areas could be seen quite clearly demarcatec [illegible]

[illegible]

alveoli contained fibrin, leukocytes, and organisms.

(*c*) *Streptococcal pneumonia* Hemolytic streptococci were frequently isolated in certain localities (e g, by Blanton and Irons, 1918, Fildes, Baker, and Thompson, 1918, Goodpasture, 1919 *a*, Opie *et al*, 1919 *b*) Goodspasture reported that hemolytic streptococci were more commonly encountered in the cases which he examined in December 1918 and January 1919 than in those occurring in the fall of 1918 Pneumonia due to the hemolytic streptococcus had a characteristic appear-

ance (see MacCallum, 1919)· there was a scanty pleural exudate, edema of the interlobular septa, while deep red consolidated areas, not sharply demarcated, were also present. Microscopically, the alveoli contained leukocytes, blood, fibrin, and streptococci, the alveolar vessels contained fibrinous thrombi. The bronchi also contained exudate and streptococci, and their epithelium was widely necrotic.

(*d*) B influenzae *pneumonia*. Pfeiffer's bacillus was frequently found in the lungs of fatal cases of influenza (e.g., by Dietrich, 1918; Simmonds, 1918, McIntosh, 1918, Opie *et al.*, 1919 *b*; Spooner, Scott, and Heath, 1919, Tytler, Janes, and Dobbin, 1919, Wolbach, 1919; Greig, 1920–1). According to MacCallum (1919), the pneumonia associated with Pfeiffer's bacillus had characteristic features, justifying the designation of "interstitial pneumonia." Pleural exudate was not usually prominent, but the surface might be covered with fibrin On section, the lung surface showed numerous yellowish peribronchial nodules, almost resembling tubercles, and purulent bronchitis was usually present. Microscopical examination of the nodules showed that the alveoli contained a leukocytic exudate with little hemorrhage. There was proliferation and infiltration of the walls of the bronchioles and alveoli

Summary. It is thus evident that numerous types of lesion might be found in influenza. In fact, the heterogeneity of the pathological pictures was one of the characteristic features of the disease. Generally speaking, in the earlier cases the lesions were mainly of a toxic nature, the lungs showing numerous hemorrhages. In the 1919 (third) wave the rôle of secondary organisms was firmly established and the lesions then produced were of a different nature. The essential changes were a necrotizing type of inflammation of the bronchi and bronchioles.

Other Organs

Purulent sinusitis was very commonly observed, and otitis media might also be present. The meninges were often edematous and the brain tissue congested Hemorrhages might be observed in the rectus abdominis, and Champtaloup and Drennan (1919) recorded a waxy degeneration of voluntary muscles. Mills (1919) reported cellular desquamation in the testis, and infiltration with giant cells

There was usually evidence of cloudy swelling or fatty degeneration in the liver, while in the kidney French (1920) found frequently a state of general vascular congestion He also drew attention to enlargement and congestion of lymph glands, especially below the bifurcation of the trachea

EPIDEMIOLOGY OF PANDEMIC INFLUENZA

The epidemiology of influenza proves an interesting study, and it is regretted that it can only be dealt with here quite shortly, and that with especial reference to the state of affairs in Great Britain Bruce Low (1920) collected the epidemiological data relative to Europe, while James (1920) did so for Australasia and the East The monograph of Burnet and Clark (1942) gives a wealth of valuable information on epidemiology.

Characters of the Various Waves of Influenza

The pandemic influenza of spring, 1918, is often known as "Spanish influenza," but it is uncertain that the disease actually broke out first in Spain. In fact, it is more probable that the earlier cases occurred in France From France, the main battle front of World War I, the disease seemed to spread all over the world In many places 3 distinct waves were experienced

First wave. In April 1918 the disease broke out in the French and British armies fighting in France In the British armies the first cases occurred at Rouen and Wimereux (Influenza Committee, 1918) The peak was experienced about June 25 and the epidemic was virtually over by mid-August In the United States the first

cases were probably seen in Chicago during the first week in April 1918, but it was not until later, when postmortem examinations were carried out by LeCount (1919 *c*), that it was realized that the condition was influenza

Great Britain was attacked rather later than France, about June The disease involved Germany and Italy slightly later Definite influenzal outbreaks were also experienced in India and New Zealand in July, although Australia escaped In August, South Africa was involved

The first wave was comparatively mild, and the term "3-day influenza" was sometimes applied, pneumonic complications were rare

Second wave This wave was very much more severe and had a considerably higher death rate The outbreak was at its height in September and October in France, Great Britain, and America, almost all parts of the world were involved in this wave

Third wave This wave occurred in February and March 1919, particularly in the United States and in Great Britain, and was the severest of the three in certain localities, in other areas, however, it was less severe than the second wave

Attack Rate

Estimates of the number of persons attacked varied considerably, but probably up to half the population of the world would be a fair estimate. The majority of cases occurred in persons under 20 years of age In the United States it has been reported that there was a higher attack rate in the poorer than the richer classes (Sydenstricker, 1931)

Spread

Weather did not play any primary part in the spread of the epidemic, at any rate in Great Britain (Ministry of Health, 1920) The disease must have been spread by droplet infection As the virus had not been isolated in those days it was not possible to say accurately for how long an influenzal patient was infective. Probably, however, the virus was only present in the nose and throat for a few days during the acute stages, and for a few hours in the incubation period

There were numerous instances where influenza broke out in communities fol- [illegible] sponsible for the spread of the disease by droplets, in certain cases, especially in institutions, infection may have been contracted from fomites, such as improperly cleansed eating utensils

Although epidemiological observations rendered it certain that infection was spread by droplets of infective secretion, experiments to test this hypothesis failed, because volunteers inoculated with the throat secretions of cases of influenza did not contract the disease (see, e g, McCoy and Richey, 1921, Rosenau *et al*, 1921).

Periodicity of the Waves of Pandemic Influenza

It has been shown how the 1918–19 pandemic was composed of 3 distinct waves Influenza was also prevalent during the few succeeding years In the 1890 pandemic also there were similar waves and recurrences Brownlee (1920, 1923) has postulated a 33-week cycle for these recurrent outbreaks, basing his hypothesis on a number of British and American statistics It may be said that the existence of this cycle has not been definitely accepted by the majority of epidemiologists.

Etiology of Influenza Pandemics

One is faced with difficulty in attempting to account for the occurrence of influenza in pandemic form on certain occasions in the world's history. It is evident

that either the host or the etiological agent may be responsible With regard to the host, in certain diseases (e g., measles, see Ch XX) *it is possible partly to explain the* occurrence of epidemics on a basis of rise and fall in the number of susceptibles Such an explanation might be offered in influenza (see Hamer, 1933–4). However, it is obvious that there is a long period of years in between pandemics, e g, in 1918 nearly 30 years had elapsed since the last pandemic. Now, as it is known that the immunity in influenza is only transient, probably lasting only a few months (*vide infra*), it is difficult to see how this theory can serve, as pandemics should occur much more often than they actually do.

Jordan (1927) has fully discussed the problem of pandemic influenza He suggests that a "pandemic strain" of virus arises with unusual characters After the pandemic it probably disappears (see, also, Picken, 1941). The characteristics of an influenza pandemic are thus assumed to be dependent on factors inherent in the causal agent, rather than the potential host, and of recent years it has been suggested that the 1918 pandemic human strain of virus did have special characteristics On the basis of serological tests it has been postulated that the 1918 human strain does not now affect man, but causes in pigs the condition of swine fever. Supported by Laidlaw and by Shope, this explanation of the observed serological data is not accepted by others, who feel that there may be another interpretation This subject is more fully discussed below (see p. 667).

Another explanation is that there was a high incidence of secondary bacterial invaders, and Shope (1944) suggested that this occurred in the second wave, the first being a pure virus infection.

Burnet and Clark (1942), who discuss in some detail the above alternatives, suggest for their own part that the 1918–19 pandemic virus, while similar antigenically to present-day virus A, possessed the unique biological property of attacking the alveoli as distinct from the bronchi, and perhaps of affecting vascular endothelium also. They consider that the abnormal conditions in France during the latter years of World War I, especially the great opportunities for transfer of infection by droplets, may have favored the emergence of a strain or strains with these highly "pneumotropic" properties There seems little doubt that secondary invaders played a definite rôle, and determined the severity of pneumonic complications Burnet and Clark suggest that by the end of 1919 the virulence of most strains was considerably reduced, probably owing to passage through partly immune persons

It is not our intention to theorize on this point any further, and we must close with an admission of present uncertainty. It does not seem that the problem can be solved unless another pandemic appears. If this unfortunate event should occur, it ought to be comparatively simple to test the new strains of virus and compare them antigenically and by animal test with known epidemic strains

IMMUNITY

There was some doubt as to whether one attack conferred resistance against a subsequent attack, and undoubtedly some reports seemed to indicate that there was a transient immunity acquired in convalescence Thus, Scoccia (1918) in Italy reported that sufferers from the May-June wave did not again contract the infection in September In India, Malone and McKendrick (1919–20) also reported an immunity lasting approximately 9 months Similar resistance to second attacks was reported by other workers (e g, Hamilton and Leonard, 1919, Speares, 1919, Waters, 1920)

A very thorough study of this question was undertaken by the United States Public Health Service in Baltimore (see Jordan, 1927) This work showed that any such immunity had quite waned after a year Thus they found that approximately 8 per cent. of persons who had influenza in 1918 were attacked in January and February 1920, and the same number of persons who had not suffered from the

1918 attack developed the disease in 1920. Similar observations were recorded by Jordan and Sharp (1920) and by Vaughan (1921)

With regard to passive immunity, certain workers reported beneficial effects following the use of human convalescent serum (see, e g, Grigaut and Moutier, 1918, McGuire and Redden, 1918, 1919, Gould, 1919, Francis, Hall, and Gaines, 1920, Sanborn, 1920).

ATTEMPTED ISOLATION OF THE ETIOLOGICAL AGENT OF PANDEMIC INFLUENZA

Although it is now almost certain that the cause of pandemic influenza was a filtrable virus, no such conclusion was reached in those days. Various theories were discussed first, that Pfeiffer's bacillus was the cause of pandemic influenza, second, that the disease was caused by a virus, pathogenic bacteria being associated as secondary invaders in certain, usually fatal, cases, and thirdly, Olitsky and Gates (1922, 1923) advanced the claims of *Bact pneumosintes*.

1. The Rôle of *B. influenzae*

Pfeiffer (1893) isolated *B influenzae* from cases of influenza and claimed it as the etiological agent. Almost from the start the primary relationship of this organism to the disease was doubted, for the following reasons

(*a*) *Distribution in influenza cases* Although during the pandemic (1918) the organism was frequently isolated from the upper respiratory tract and from the lungs (*vide supra*), a number of careful observers either failed to isolate it from genuine cases, or else found it to occur only in small numbers in the cultures (e g, Bernhardt, 1918, Blanton and Irons, 1918, Influenza Committee, 1918, Mandelbaum, 1918, Camp Lewis Pneumonia Unit, 1919).

(*b*) *Distribution in other conditions.* One of the most serious and old-standing objections to accepting Pfeiffer's bacillus as the primary agent of influenza has been that it is often found in other conditions (see Leiner, 1901, Rosenthal, 1905, Wollstein, 1906) During the pandemic numerous authors found it in the throats of normal persons, in some cases even up to 40 per cent. (see, e g, Fildes, Baker, and Thompson, 1918, Kinsella, 1919, Lord, Scott, and Nye, 1919, Opie *et al*, 1919 *a*, *b*, Pritchett and Stillman, 1919, Scott, 1922)

(*c*) *Serological structure of* B influenzae There was great unanimity regarding the antigenic structure of this organism, which was extensively investigated (Fleming and Clemenger, 1919, Park, Williams, and Cooper, 1919, Park and Williams, 1919, Rapoport, 1919, Valentine and Cooper, 1919, Bell, 1920, Small and Dickson, 1920, Coca and Kelley, 1921, Jordan and Sharp, 1922, Scott, 1922). These workers showed that there were numerous different types, and the pandemic, or even small localized sections of it, was definitely not caused by strains serologically identical. In fact, a number of different strains might be isolated from one person These findings could not be [illegible]
of influenza It is now [illegible]
there is a number of [illegible]

(*d*) *Pathogenicity* [illegible]
[illegible]

fluenzae for man, certain volunteers have been inoculated in the throat with cultures Some have reported that influenza did not follow such inoculations (Lister and Taylor, 1919, Rosenau, 1919, Yamanouchi *et al*, 1919, Ishiwara, 1923). Others have reported slight rises of temperature, with pains and perhaps respiratory symptoms, and even some prostration (Davis, 1919, Cecil and Steffen, 1921, Park and

Cooper, 1921). These attacks, however, were not nearly so severe as typical influenza

Recently Smorodintseff *et al.* (1936 *a*) have carried out a carefully controlled series of experiments in Russia. They inoculated human volunteers with cultures of Pfeiffer's bacillus, and after 4 to 6 hours noted the development of coryza with slight rise of temperature. These symptoms soon subsided and were definitely not those of a genuine influenza.

It is probable that *B. influenzae* can cause a purulent bronchitis (Mulder, 1940 *b*)

Conclusions. We can only conclude that Pfeiffer's bacillus was not the causal agent of pandemic influenza. In like manner, it is definitely not the cause of epidemic influenza. This is not to deny any rôle to Pfeiffer's bacillus, for during the pandemic it was undoubtedly of importance as a secondarily invading organism

2. The Virus Theory

Many bacteriologists began to feel that *B. influenzae* was not the causal agent of pandemic influenza, and in the absence of any other organism occurring regularly, suspicion fell on the possibility of a filtrable virus being present. Many experiments were carried out which did little or nothing to support this theory, because filtrates of nasopharyngeal secretion of cases did not produce definite influenza in inoculated volunteers (see, e.g., Keegan, 1918; Lister and Taylor, 1919; Rosenau, 1919, *et al.*, 1921, McCoy and Richey, 1921).

Moreover, it must be admitted that even today, when the virus theory of influenza is generally accepted, filtrates of nasopharyngeal secretion may not transmit influenza. Thus Dochez, Mills, and Kneeland (1936) only produced a common cold in certain experiments

However, certain workers obtained more positive results Nicolle and Lebailly (1919) claimed to have transmitted influenza to 2 persons by a subcutaneous inoculation of filtered sputum The same workers also claimed positive inoculations of monkeys by the nasal route. In Japan, Yamanouchi *et al* (1919) claimed to have transmitted the infection to a number of volunteers by injection of filtered and unfiltered sputa intranasally and subcutaneously, blood of acute cases was also said to prove infective. Selter (1918) and Leschke (1919) also reported somewhat similar results

Gibson, Bowman, and Connor (1918, 1919), and guinea-pigs, reported the production of ıfluenza, after inoculation with filtered and her, they reported that their "virus" could be cultured in Noguchi's medium, when a growth of a minute coccus was obtained In view of our present knowledge of the properties of the influenza virus, *it is* most unlikely that these workers really isolated the causal agent.

In conclusion, it is evident that the virus theory, though attractive, was not supported by much experimental work, and probably derived its main support from the fact that no cultivable organism seemed to be responsible.

3. The Rôle of *Bacterium Pneumosintes*

This coccobacillus is a small filter-passing organism which was isolated from the nasopharyngeal washings of cases of catarrh of the upper respiratory tract by Olitsky and Gates (e g, 1922, 1923) Although it is probably a specific organism it is unlikely that it is causally related to influenza (for further information see McCartney, 1929)

CHAPTER LV

EPIDEMIC INFLUENZA

Introduction

In 1933 many believed human influenza to be a virus disease, but this theory rested on no certain foundations. Progress was hindered by the lack of an animal to which the disease could be readily and economically transmitted Accordingly, the widest interest was taken in a paper published from the National Institute of Medical Research, London, by Smith, Andrewes, and Laidlaw (1933).[1] They reported the susceptibility of the ferret to a filtrable virus present in the nasopharyngeal secretions of typical cases of epidemic influenza. As the filtrable virus was recovered from a number of cases there appeared little doubt that it was actually the cause of epidemic influenza, especially as it was not isolated from sporadic or vague "influenzal" cases Later it was reported that mice could be infected with this virus, thus considerably facilitating research (Andrewes, Laidlaw, and Smith, 1934).

Francis (1934) in the United States was able to confirm the presence of such a virus in the sputum of cases in Puerto Rico Later (1934–5) he found the virus in New York and Philadelphia (see also 1935) Since this time many workers have isolated strains of virus from cases of epidemic influenza, and it is now universally accepted as the etiological agent.

Following the suggestion of Horsfall *et al* (1940), influenza virus strains are separated into 2 main types, the original or Type A, which includes the well-known WS and PR8 strains, and the more recently discovered Type B including the Lee and TM strains These types resemble each other closely in biological properties but can be differentiated antigenically.

Very remarkable advances have been made in influenza research of recent years For example, it has been found that the virus grows readily in the allantoic and amniotic sacs, in the former in such abundance that it can be obtained in large enough quantity for vaccine production It has also been shown that virus causes agglutination of chick and other red cells, and that sera containing influenzal antibody inhibit this agglutination

A condition closely resembling human influenza has been observed in swine since 1918 This disease, known as swine influenza, occurs commonly in the Middle West of the United States Although the condition does not, so far as is known, affect man, it presents so many analogies to the human disease, and the causal viruses are so closely related, that it will be described below in some detail. It has, moreover, been suggested that the present virus of swine influenza is the modern survivor of the 1918 pandemic human strain

A number of general reviews on the subject of influenza has appeared of recent years (Laidlaw, 1934, 1935, Shope, 1936 *c*, *d*, 1944; Andrewes, 1937, 1939, 1942 *b*, Béclère, 1937, Francis, 1937, 1938, 1941 *a*, 1947 *b*, Hallauer, 1937, Burnet, 1943 *c*, Parodi and Vilches, 1944, Stuart-Harris, 1940, 1945 *a*, *b*, 1947), Burnet and Clark (1942) have published a particularly valuable monograph.

Swine Influenza[2]

The disease was first noticed in the Middle West of the United States of America in 1918, affecting many stocks of pigs in the Iowa district, with a high mortality. At the same time, the pandemic of human influenza was raging, and Koen suggested that the two diseases might be the same (see Dorset, McBryde, and Niles, 1922–3, also McBryde, 1927)

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

[2] Much additional information about the swine influenza virus will be found in the following chapters

Koen suggested that the pigs were infected initially from human beings Of recent years this suggestion has been amplified, and it is suggested that the original 1918 human strain no longer exists in man, but only in the pig, as one of the causal agents of swine influenza Shope has shown that the malady is caused by a synergism of a filtrable virus and a hemophilic bacterium

Swine influenza occurs also in Europe, Great Britain, and N Ireland, and apparently organisms other than *H. suis* may occur along with the virus (Blakemore and Gledhill, 1941; Glover, 1941 *a;* Glover and Andrewes, 1943).

Features. The features in pigs are virtually identical with those produced by the combined injection of the 2 causal agents under experimental conditions. The disease occurs mainly in the autumn and early winter. The onset is sudden and many animals die, the course is manifested by fever, cough, and prostration (see Shope, 1931 *a*).

Pathology Swine influenza is characterized pathologically by an exudative bronchitis and bronchiectasis (Shope, 1931 *a*, 1936 *c*). There is massive pulmonary atelectasis with pneumonic consolidation Microscopically, a polymorph exudate fills the bronchi and bronchioles, the lining membrane of the bronchial tree is severely affected and the cilia are lacking or damaged in many areas The cervical and bronchial lymph glands are edematous and enlarged

Isolation of the etiological agents. The etiology of swine influenza has been elucidated by Shope in a comprehensive series of investigations (Shope, 1931 *a*, *b*, 1932, 1934 *a*, *b*, 1935, 1936 *b*, *c*, 1937 *a*, *b*, *c*, 1938, Lewis and Shope, 1931; Orcutt and Shope, 1935). Shope has shown that the disease can be transmitted readily by contact between infected and healthy pigs or more directly by instillation of infective material intranasally The infective element consists of the virus of swine influenza and *Hemophilus influenzae suis;* the former is the most important and can produce a mild catarrhal condition *per se*

Hemophilus influenzae suis This gram-negative hemophilic organism is constantly present in association with the virus, by itself it has only a feeble pathogenicity for pigs Swine strains are antigenically heterogeneous. Known human strains of *H. influenzae* (meningeal or respiratory) show no synergistic effect on swine virus (Mote and Fothergill, 1940)

Swine influenza virus. By itself this produces in pigs a mild attack of swine fever (filtrate disease), but with the bacterium the experimental disease is severe and resembles the natural condition The virus is pneumotropic and can be isolated from the turbinates, tracheal exudate, and lungs of infected pigs, it does not occur in the blood or viscera (Orcutt and Shope, 1935). The virus is pathogenic for the ferret, and pneumonia develops when inoculated under ether

The virus is also pathogenic to mice, and may be isolated directly from cases of swine influenza by inoculation in these animals without preliminary ferret passage. Pneumonia usually follows nasal instillation of virus Strains vary somewhat in the ease with which they become adapted to mice (Shope, 1935)

When *H suis* and swine virus are inoculated together on the chorio-allantois, a higher proportion of embryos is killed than by either component separately This enhancing effect is produced equally well by old laboratory cultures, heat-killed organisms, or filtered extracts of frozen and dried organisms (Bang, 1943, *a*, *b*)

It is thus seen that the properties of the virus of swine influenza closely resemble those of human influenza The question of the antigenic relationship of the two viruses is discussed elsewhere (see p 587), where it will be seen that it is very close

Passage The disease can be maintained under experimental conditions by subjecting fresh animals to contact infection with a declared case, both the bacterium and virus are transferred, and eventually a typical attack of swine influenza develops Shope (1934 *a*) records an experiment where he had been passing a strain of swine influenza in this way when it suddenly became less contagious, it was found that only the virus was now being transferred, the disease being the mild form due to this agent alone (filtrate disease)

Survival of virus in nature Shope (1941 *a*) found that multiple intramuscular injections of *H suis* precipitated the double infection in apparently normal swine, suggesting that the animals were harboring the virus in a latent condition He found (1941 *b*) that the swine lung worm acts as an intermediate host, carrying the virus in a "masked" noninfective form To produce infection, a stimulant, such as injections of *H suis,* is needed Infection cannot be provoked in the summer months The virus can persist in the lung worm for at least 2 years. Swine carrying infected lung worms develop pneumonic

consolidation most markedly around the worms In certain cases, after provocative injections, infested swine become immune, but not all; in these cases the worms are probably not situated in the respiratory tract Later (1943 *a*) he reported finding virus in lung worm ova obtained from the respiratory tract or feces of infected or convalescent swine. Masked virus persists for over one year in lung worm larvae in the earthworm *Allolobophora caliginosa f typica* (Savigny) and *f. trapezoides* (Dugès). Shope (1943 *b*) found masked virus in lung worm larvae from earthworms dug up in the Middle West. There is no doubt from these researches that the swine lung worm is the reservoir of swine influenza virus infection, and the intermediate host in the field.

[illegible] recovery from infection, "filtrate disease" (i e, the [illegible] both the virus and the [illegible] ne influenza, animals are [illegible] sistance to infection after vaccination, injections of the bacterium alone confer only a partial immunity capable of modifying, but not preventing, the course of a subsequent attack of swine influenza (Shope, 1937 *b*). Injections of tissue containing virus are effective, and swine fever can be prevented by inoculation of infected ferret lung given intramuscularly or subcutaneously. (For a paper on the development of immune bodies in infected swine, see Rosenbusch and Shope, 1939)

Ferrets can be immunized by subcutaneous injections of infective homologous lung, mouse or pig lungs do not, however, achieve this result (Shope, 1936 *b*). Mice also can be immunized subcutaneously by injection of homologous lung, ferret or pig lung is not effective (Shope, 1936 *b*). However, if the intraperitoneal route is used for immunization, then ferrets and mice can be immunized by virus-infected tissue from any of these 3 animals

Equine influenza Although this disease is caused by a hemolytic streptococcus and filtrable virus, there is no evidence that the virus is related to human or swine strains of influenza (Jones and Maurer, 1942, 1943).

Calf influenza An influenzal pneumonia with enteritis occurs in calves. The etiology is uncertain, but it appears to be due to a filtrable virus, probably together with a hemophilic organism (Gilmore, 1939, Lamont and Kerr, 1939, Baker, 1943).

CLINICAL FEATURES OF HUMAN INFLUENZA

The signs and symptoms of influenza proved by laboratory tests to be due to virus A or B are usually fairly characteristic, although infections can range in severity from those that are only subclinical to fatal pneumonias.

It is useful to use the term "clinical influenza" for cases occurring in a proved epidemic and diagnosed on clinical grounds alone (Horsfall *et al*, 1940). In any influenza epidemic, only a proportion of cases of clinical influenza will show laboratory evidence of infection. The term influenza Y is given to cases clinically typical, but apparently due to infection with an unknown agent.

There is the additional difficulty that there are a number of other infections of the respiratory tract, not unlike influenza clinically, but of unknown etiology The term "febrile catarrh" has been used for these cases (Stuart-Harris, 1937 *a*, *et al*, 1938, Wauchope, 1935), but it is not really possible to achieve a differentiation from influenza on purely clinical grounds (Stuart-Harris, 1945 *a*). These catarrhs are probably caused by Pfeiffer's bacilli, streptococci, and pneumococci (Thomson and Thomson, 1935), and sometimes by the virus of the common cold (Stuart-Harris, 1945 *a*), or the viruses of atypical pneumonia American workers have drawn attention to a condition they call "catarrhal fever" (*U.S Nav. Lab. Res Unit*, 1944 *c*) The differential points from influenza are the insidious onset of catarrhal fever, the predominance of respiratory as opposed to constitutional symptoms, the productive and paroxysmal cough, and the frequency of pharyngitis and tonsillitis with exudate (see also Stuart-Harris *et al*, 1938).

Unless complete laboratory tests are carried out, cases will be labelled as influenza that are not Further, Ziegler *et al* (1947) have shown that double infection with atypical pneumonia and influenza may occur.

It should not be necessary to give a detailed description of the features of the common type of infection due to virus A, but it is useful to have some indication as to what constitutes "typical influenza" Stuart-Harris (1945 *a*) gives the following description of a typical case in an adult:

On the first day the patient has a headache, feels shivery and ill. He sleeps poorly and wakes at intervals with a dry cough. Aching in the back and limbs is common, and so are dizziness, vomiting, and fainting. The temperature rises to 102° F., and there is a frontal headache, anorexia, and drowsiness. The face is flushed and the lips cyanosed. On the following day the temperature falls, but the cough persists. On the 3rd day there is renewal of fever, and the cough continues The fever usually subsides after 3–5 days. Stuart-Harris refers to atypical attacks, in which there may be a premonitory cold, vomiting, acute coryza, a sore throat, or aphonia. Some cases are afebrile, while others show a diphasic fever. Pharyngitis was found to be a characteristic feature in an outbreak of A infection, and pharyngeal swabs showed destruction of epithelium, and mononuclear exudate (Adams, Pennoyer, and Whiting, 1946).

There are probably no significant differences between influenza A and B, but the onset of the latter may be more insidious, the illness, milder, and the symptoms less uniform, the infection can vary in severity from a coryza to a severe attack, and nausea and vomiting may be relatively frequent (Taylor *et al*, 1942–3, Hare, Stamatis, and Jackson, 1943, Beveridge and Williams, 1944, Stuart-Harris, 1945 *a*, Jackson, 1946, Hirst *et al*, 1947). Premonitory symptoms of coryza have been noted in some outbreaks, but have been absent in others.

Comparison with Pandemic Influenza

Epidemic influenza is very much less severe than pandemic, the attack rate and death rate being much lower. With regard to clinical features, the uncomplicated cases are remarkably alike At the present day, fortunately, there is much less tendency for respiratory complications to develop An interesting report is that of Doull and Bahlke (1933), who had the opportunity of observing nurses in the 1918–19 and the 1928–9 outbreaks In 1928–9 pneumonia was rare and the disease seldom proved fatal. Both outbreaks were characterized by leukopenia, muscular pains, and pyrexia, but these were distinctly less prominent in 1928–9

Influenzal Encephalitis

Greenfield (1930, and see *Report*, 1930) drew attention to the occurrence of 3 cases of acute disseminated encephalomyelitis following attacks of influenza. Clinically the picture was of a severe paraplegia with incontinence of urine and feces. Histologically there was found perivascular demyelination and cellular infiltration, exactly as in similar cases following on measles and vaccination This question is further discussed in the section on postvaccinal encephalitis (see Ch. XXXV).

Leigh (1946) in London observed a small series of cases of encephalitis, myelitis, and polyneuritis, occurring at the peak of the 1946 influenza B outbreak Although it was not possible to be certain, it appeared likely that most of the cases suffered from influenza a few days before the onset

Broun *et al* (1945) isolated an influenzal type of virus from the CSF of a case of encephalitis developing 6 weeks after a respiratory illness resembling mild influenza

Woodside Throat

There is some suggestion that an acute throat infection seen in Australia may be due to influenza virus (Mawson, 1942).

DISTRIBUTION OF VIRUS IN THE BODY

Virus is most easily recovered from nasopharyngeal washings, or gargling, nasal secretion may also prove infective. Virus is usually present for the first few days

of the illness only, but in 1 case it was found as late as the 7th day (Francis *et al*, 1937 *a*) Virus has also been recovered from the throat of healthy contacts, and in 1 case 24 hours before the onset of influenza (Francis *et al*, 1937 *a*, Crowley, Thigpen, and Rickard, 1944).

BACTERIOLOGY OF UNCOMPLICATED INFLUENZA

Hoyle and Fairbrother (1937 *a*) failed to find pathogenic organisms in specimens yielding influenza virus, but pneumococci and *H. influenzae* may occur (Stuart-Harris *et al*, 1938). In general, pathogenic bacteria are found more readily in cases complicated by pneumonia (see below).

Cases of influenza nursed in general wards are liable to cross infection, and Cruickshank and Muir (1940) have described a series of cases of secondary infection of the upper respiratory tract due to hemolytic streptococci.

RESPIRATORY COMPLICATIONS OF INFLUENZA

It is now generally believed that the virus of influenza (A or B) is itself responsible for the ordinary uncomplicated case of epidemic influenza The virus depresses the resistance of the respiratory tissues and opens the way for secondary invasion by organisms from the throat Recent reports have shown that staphylococci and streptococci may be isolated from the lung, as they were in 1918–19 Although the virus itself produces lesions in the respiratory tract of man, as it does in animals, it appears that fatality is largely determined by secondary infection and the development of pneumonia Pneumonia usually comes on within 14 days of the onset of influenzal symptoms

Scadding (1937) has formulated the following views, based on his correlation between the clinical and pathological features of cases (influenza A) occurring in 1936–7. He regards the influenza virus itself as capable of causing a descending infection of the respiratory tract, which may progress to any level In the more severe cases, however, the process is modified because of secondary bacterial invasion. He regards the fulminant cases as due to the combined action of the virus plus a virulent bacterium The virus acts by lowering the resistance of the tissues to invasion by Pfeiffer's bacillus, streptococci, staphylococci, or pneumococci, and it is due to these organisms that fatalities occur

As Scadding points out, there is an analogous condition in poison-gas pneumonia where the [illegible]

severe attack

Distribution of Virus and Bacteria in Respiratory Complications

In certain cases influenza virus can cause a fatal pneumonia, and there have been reports of the isolation of influenza virus A by itself from the lungs of such cases (Finland *et al*, 1945; Parker *et al*, 1946). McIntosh and Selbie (1937 *a*, *b*) isolated an influenzal type of virus from the lungs of 2 fatal cases, the virus had somewhat unusual properties

According to Stuart-Harris (1945 *a*), pneumococci, either in pure culture or associated with other organisms, are the commonest bacteria occurring in pneumonia following influenza A (see also Scadding, 1937, Stuart-Harris *et al*, 1938, Nigg *et al*, 1942) Jackson (1946) described a series of cases of pneumococcal pneumonia in an epidemic of influenza B

Hemolytic streptococci are unusual secondary invaders, but have been reported as occurring in pure culture or associated with other organisms (Watjen, 1937, Davis, 1944)

Nonhemolytic streptococci have been reported (Emdin, 1942)

Staphylococcus aureus is the most serious secondary invader, and there has

been a number of reports of its isolation from the bronchi or lungs of fatal cases of influenzal pneumonia. Thus this organism has been isolated from the lungs of cases of pneumonia occurring during influenza epidemics (Burgess and Gormly, 1930; Scadding, 1937; Finland *et al.*, 1942; Michael, 1942; Wollenman and Finland, 1943).

More definite evidence of the association of *Staph. aureus* and influenza virus has been obtained by workers who have isolated virus A and the organism from the bronchi or lungs at the same time (Stuart-Harris *et al*, 1938, Stokes and Wolman, 1940, Parker *et al.*, 1946). Influenza virus B and *Staph. aureus* have also been recovered from the bronchi or lungs in influenzal pneumonia (Himmelweit, 1943; Burnet, Stone, and Anderson, 1946).

Staph. aureus is more usually present in pure culture, but may be associated with pneumococci, *H. influenzae*, or hemolytic streptococci (Wollenman and Finland, 1943; Davis, 1944; Parker *et al.*, 1946).

PATHOLOGY

In general, the changes in epidemic influenza are similar to those in pandemic influenza, described fully above.

Virus pneumonia. There is no doubt that in certain cases influenza virus itself produces a fatal pneumonia, without the presence of secondary invaders The virus can give rise to a range of pathological lesions from tracheitis and pharyngitis, to bronchitis, bronchiolitis, and bronchopneumonia. The pathological features of the lesions due to the virus alone have been described by various authors (McCordock and Muckenfuss, 1933; Scadding, 1937, Parker *et al*, 1946).

Thus, McCordock and Muckenfuss found that the interstitial bronchopneumonia of influenza closely resembles "virus pneumonia" that can be produced in animals with vaccinia. The earliest influenzal lesion in the lungs is a hemorrhagic and edematous lobular [illegible] lar and alveolar ep[illegible] Later, an interstiti[illegible]e and a nonpurulent exudate in the alveoli Gray carried out the pathological examination of Scadding's cases (*vide supra*). He found an interstitial inflammation and some necrosis of the alveolar walls with resultant hemorrhage. Necrosis of the tracheal and bronchial epithelium was also prominent.

In the past a necrotizing bronchiolitis has been regarded as characteristic of influenzal pneumonia in man. Parker *et al.* did not, however, find this change in cases apparently due to the virus alone. They suggest that this particular lesion is due to invasion by staphylococci (see below). They found edema, alveolar hemorrhages, fibrin exudate, and formation of a hyaline membrane The bronchiolar epithelium was intact, but the walls were infiltrated with leukocytes, the lumina contained mucus, leukocytes, red cells, and fibrin

Pneumococcal pneumonia Influenzal pneumonia associated with the presence of pneumococci usually has the pathological features of lobar pneumonia It is possible that a number of small localized "epidemics" of pneumococcal pneumonia may, in reality, be secondary to influenzal infection For example, a Type I outbreak was subsequently shown by serological tests to be associated with infection due to virus B (Commission, 1945).

Streptococcal pneumonia Watjen (1937) examined cases of influenzal pneumonia associated with the presence of hemolytic streptococci He found a widespread hemorrhagic and inflammatory involvement of the respiratory passages and lung tissue similar to that seen in 1919

Staphylococcal pneumonia At the present day, the majority of fatal cases of influenzal pneumonia seems to be associated with *Staph aureus* The pathological features of acute infection with *Staph aureus* and virus A have been described (Stokes and Wolman, 1940, Wollenman and Finland, 1943, Parker *et al*, 1946)

Finland *et al* (1942) who observed 66 cases suggested that under certain conditions the organism assumed an epidemic spread together with the virus They found that staphylococcal infections varied from a simple tracheobronchitis to a fulminating acute and fatal hemorrhagic pneumonia.

The lungs usually show a bilateral involvement most marked in the posterior and dependent portions. Multiple abscesses related to the bronchi and bronchioles are common.

The trachea, bronchi, and bronchioles have lost most of their epithelial lining in a necrotizing process, and their walls are infiltrated with inflammatory cells and much fibrin. A hyaline membrane may be found. The alveolar capillaries are congested and the alveoli edematous, and contain red cells and polymorphs Sometimes the picture is more of an acute hemorrhage than an infection.

Chronic lesions may also occur in infections with virus A and *Staph aureus* (Wollenman and Finland, 1943). There are large bronchiectatic cavities with firm fibrous walls There is some regeneration of the bronchial mucosa. The alveolar lining may be replaced by a low cuboidal epithelium Alveolar exudate shows evidence of organization

The pathological features of acute infection with *Staph aureus* and virus B resemble those described for virus A. Thus Himmelweit (1943) reports a case in which the lungs showed blood-stained edema, with patchy peribronchial consolidation. The epithelial lining of the trachea and bronchi was shed, and the lungs showed a patchy bronchopneumonia.

It has been suggested that in addition to respiratory complications, virus A may cause myocarditis (Finland *et al*, 1945).

MORTALITY

It will be recalled that during t[illegible] deaths occurred in young adults Under [illegible] influenza mortality statistics since 190[illegible] disappear, but that it took about 10 [illegible] was again present, that is to say, before the maximum number of deaths again occurred in those over 60 years of age. The majority of epidemics occurs in December and January, and Spear (1934) has shown that the week of maximum deaths (in London) is from the 8th to the 10th week of the year, i e, in March) In a large country the total mortality from epidemic influenza remains considerable, thus, in the United States of America, Collins (1930) reported that in the 1928–9 epidemic about 50,000 deaths occurred from influenza and pneumonia (in excess of expected seasonal rate) In the 1920 epidemic 100,000 deaths occurred, while in the epidemics of 1922, 1923, 1926, and spring 1928 there were 150,000 deaths. (For a detailed study of the influenza and pneumonia mortality from 1910 to 1944 in America, see Collins *et al*, 1930, Gover, 1943, Collins, 1944, 1945.)

Mortality from influenzal pneumonia is still very high, thus Collins (1934), studying the American 1928–9 epidemic, found that the fatality rate of pneumonic cases varied from 10 per cent to 33 per cent

Epidemic influenza has a noticeable effect in increasing the mortality from other respiratory diseases in which it is not actually mentioned as a contributory cause of death Thus Stocks (1935), studying the statistics from 1921 to 1933, found that whooping cough had the highest rise. Not only respiratory diseases, but also diseases of the heart and nervous system, showed an increased mortality due to influenza epidemics

EPIDEMIOLOGY

The term "epidemic influenza" should be restricted in epidemiological discussions to widespread prevalences producing a definite excess mortality from influenza and pneumonia in large populations, the term "outbreak of influenza"

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Thus, McCordock and Muckenfuss found that the interstitial bronchopneumonia of influenza closely resembles "virus pneumonia" that can be produced in animals with vaccinia The earliest influenzal lesion in the lungs is a hemorrhagic and edematous lobular consolidation Microscopically there is necrosis of the bronchiolar and alveolar epithelium and walls, with coagulum and red cells in the alveoli Later, an interstitial pneumonia follows with infiltration of the interstitial tissue and a nonpurulent exudate in the alveoli. Gray carried out the pathological examination of Scadding's cases (*vide supra*) He found an interstitial inflammation and some necrosis of the alveolar walls with resultant hemorrhage. Necrosis of the tracheal and bronchial epithelium was also prominent

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Staphylococcal pneumonia At the present day, the majority of fatal cases of influenzal pneumonia seems to be associated with *Staph aureus* The pathological features of acute infection with *Staph aureus* and virus A have been described (Stokes and Wolman, 1940, Wollenman and Finland, 1943, Parker *et al*, 1946)

TABLE 23

MAIN REFERENCES TO OCCURRENCE OF INFLUENZA B IN RECENT YEARS

1936	U. S. A	Francis (1940 *b*, *d*, 1941 *b*).
1938–1941	Australia	Burnet (1942 *a*).
1939	England	Lush, Stuart-Harris, and Andrewes (1941)
	U S A.	Nigg *et al* (1942).
1940	Argentine	Sordelli, Taylor, and Parodi (1941–2), Taylor *et al*, (1942–3), Vilches *et al*. (1943 *a*)
	U S A	Eaton and Beck (1941); Francis (1940 *b*, 1941 *b*), Lennette *et al* (1941), Pearson *et al* (1941).
1941	Argentine	Taylor *et al* (1942–3), Vilches *et al* (1943 *b*).
	U. S A	Reyersbach, Lenert, and Kuttner (1941).
1942	England	Stansfeld and Stuart-Harris (1943)
1943	Australia	Beveridge, Burnet, and Williams (1944), Beveridge and Williams (1944)
	Canada	Hare, Hamilton, and Feasby (1943), Hare, Stamatis, and Jackson (1943)
	England	Stuart-Harris, Glover, and Mills (1943)
1945	Australia	Burnet, Stone, and Anderson (1946).
	Bahamas	Jackson (1946)
	Colombia	Weir (1945).
	Hawaii	Schwartz *et al* (1946).
1945	U. S A	Salk *et al* (1945 *b*), Francis, Salk, and Brace (1946), Brace and Slavin (1947), Hirst *et al* (1947), Kalter and Chapman (1947), Norwood and Sachs (1947)
	World-wide	Andrewes (1946)
1946	England	*Brit med J*, 1946, *i*, 209, UNRRA (1946); Hoyle (1946), Dudgeon *et al* (1946), Jackson (1946)
	France	Lépine, Sautter, and Reinié (1946)
	U S A	UNRRA (1946)

and Collins, 1944, Dudgeon *et al.*, 1946) Numerous other references will be found in the Tables.

The recent history of influenza in those countries where it has been most studied is as follows, information about these countries as well as others being given also in the tables.

British Isles In 1933 and 1937 there were many epidemics apparently due to virus A, which was readily isolated by ferret inoculation. Influenza was prevalent in 1934, 1936, 1938, 1940, and 1942, but there was little evidence of the presence of virus A This virus was isolated in 1935, 1939, and 1941, but was not easy to establish in ferrets or mice (Andrewes, 1942 *c*). In 1943 there were at first sporadic cases, then outbreaks, and then a widespread epidemic of A infection, the virus being readily demonstrated by ferret inoculation. Influenza A was not found in 1944 or 1945.

Infections due to virus B occurred in 1939, 1943, and 1946. Britain and N. America usually suffer from influenza epidemics at more or less the same time.

N. America. No less than 16 widespread epidemics of influenza occurred in the U. S A. between 1920 and 1944 These epidemics have been accounted for on the basis of infection with influenza A occurring every 2–3 years, and influenza B every 4–6 years (Commission, 1946 *a*). Thus these authors suggest that A infections occurred in 1923, 1926, 1929, 1931, 1933, 1935, 1937, 1939, 1941, and 1943, B infections probably occurred in 1922, 1928, 1932, 1936, 1940, 1945, and 1946. The B outbreaks of 1936 and 1940 were similar epidemiologically to A infections. Since 1940, B infection has been sporadic, but at the end of 1945 there were again outbreaks.

Australia Melbourne experienced major waves of A infection in 1935, 1939, and 1942. Sporadic cases were recognized in 1943 and 1945.

should be restricted to localized concentrations which do not cause an appreciable effect on the mortality of large populations (Commission, 1946 *a*).

Geographical Distribution

The last pandemic ended in 1919, and during the next 10 years major influenza epidemics occurred in 1920, 1922, 1926–7, 1928–9 and 1930–1, in many parts of the world. Information on world distribution of influenza has been furnished by the *League of Nations Epidemiological Reports* (1931, 1933, 1935) and various other sources (Collins, 1930, 1944, 1945; Collins and Gover, 1932–3, 1935, Doull and Bahlke, 1933; Huber, 1933; Rösler, 1933; Ganz, 1934, Reiter, 1934, Shepard, 1934; Underwood, 1934; Eckardt *et al.*, 1936, Gover, 1936, Syssine, 1936, Danson, 1937, Dennig, 1937, *Brit. med. J.*, 1937; Lancet, 1937, Meier and Baland, 1937, Stuart-Harris *et al.*, *1938, Stuart-Harris, Smith, and Andrewes, 1940, Stuart-Harris, 1941,* 1943, 1945 *a*, Andrewes, 1942 *c, et al*, 1941, Andrewes and Glover, 1944, Holland

TABLE 22

MAIN REFERENCES TO OCCURRENCE OF INFLUENZA A IN RECENT YEARS

1933–1937	*Australia* Canada England Germany Russia U. S A	See page 587
1938	Hungary U S A	Taylor and Dreguss (1941) Francis (1941 *b*)
1939	Argentine Australia Canada Denmark England Japan U S A	Vilches *et al* (1943 *a*) *Burnet and Lush (1940 a), Burnet, Stone, and Anderson (1946)* Hare, Auger, and McClelland (1942) o)
1940	Argentine Canada *England* U S A	Sordelli, Taylor, and Parodi (1941–2), Taylor *et al* (1942–3) , (1941) k, and Douglass
1941	England Australia	Andrewes *et al* (1941) *Rudd* (1941)
1942	Argentine Australia	Vilches *et al* (1943 *a*) Burnet (1943 *c*, *et al*, 1942), Burnet, Stone, and Anderson (1946)
1943	Australia Canada England Iceland S America U S A	Burnet (1943 *c*), *Beveridge, Burnet, and Williams (1944), Beveridge* ner and Scott (1944 *a*) Andrewes and Glover (1944), Sigurdsson and Bjarnason (1946) Garcia (1943) Adams, Thigpen, and Rickard (1944), Farmer and Streeter (1944), Salk, Menke, and Francis (1944), *U S Nav Med Lab* (1946), Commission on *Respiratory Diseases* (1946 *d*)
1944	Denmark	Magnus and Magnus (1944)
1945	Australia England U S A	Burnet, Stone, and Anderson (1946) *Hoyle and Lyell (1946)* Finland *et al* (1947, 1948 *a*, *b*)
1946	U S A	Forster and Shaughnessy (1946)
1947	U S A	*Trimble* (1948)

develop antibody response. There is no reason, however, why such cases should not yield virus from washings.

Incidence of Attack

A usual attack rate in the more severe epidemics has been between 10 and 20 per cent. of the populace. Thus, Huber (1933) studied 5 epidemics in soldiers in New York, occurring in 1922, 1926, 1928, March 1932, and December 1932. He found that in the 1st epidemic 9 5 per cent of the population was affected, but 20 per cent. in the 5th. Underwood (1934), studying over 17,000 persons in Leeds (England), found an attack rate of approximately 16 per cent. In the 1936–7 epidemic in London, Blood (1937) reported an attack rate of 19 1 per cent. in factory workers.

in children under 10. There has been no true peak in the young adult group, such as characterized pandemic influenza, since 1922 (see Collins, 1945)

Method of Spread

The main sources of infection are cases of the disease. Cases are probably infective in the late incubation period as well as during the first few days of illness. There is evidence that subclinical infection can occur naturally; such persons show no symptoms of infection, but develop an increase in virus neutralizing antibodies These people presumably can disseminate virus to other more susceptible persons

Influenza is essentially a droplet-spread aerial infection, and mice and human beings can be infected experimentally by inhalation. Virus can persist in air for some hours, and it has been shown experimentally that it persists for a much longer time in a dry atmosphere than a moist one (Loosli *et al*, 1943, and see Ch. LX). Fomites may play a part in transmitting infection. Virus can probably survive long enough on drinking and eating utensils to infect other persons, if the "washing-up" process is inadequate, mucoid material seems to preserve infectivity (see p 625). Virus can also survive in dust, fabrics, and materials (see p 625).

Hare and Mackenzie (1946) on the basis of experiments with *B. prodigiosus* suggest that important sources of infection are clothing and objects in the vicinity of the patient. The majority of exhaled particles falls downward onto the clothing and may be shaken off later, to be dispersed in the atmosphere, giving rise to infection in other persons These authors agree that infection may occur more directly by forcible expulsion of droplets as in sneezing, but suggest that indirect infection by dispersal of dried secretions is more likely It has been shown experimentally that sweeping the floor of a room in which virus has been atomized increases the infectivity of the atmosphere for mice (Loosli *et al*, 1943).

Virus may be spread by direct contact, as it can survive on the skin for several minutes (see p 628).

a serious outbreak affecting Eskimos 2 months after the annual visit of a trading ship Similar observations have been made in Newfoundland (Smyth, 1947) In lonely islands and other areas where communication is very limited, the infection may be spread by farm hands and others who move about from community to community (see Cheverton, 1937).

In Alaska, Pettit, Mudd, and Pepper (1936) could definitely trace the spread to lines of air travel Smillie (1930) reported an interesting outbreak in Labrador in 1928 The affected community was absolutely isolated from the outside world except through the visit of one member to a trading station This member returned

B infection occurred between 1938 and 1941, and probably between December 1941 and January 1942; this infection is probably endemic (Beveridge and Burnet, 1944). Sporadic B infections occurred in 1943 and 1945.

Differences in Behavior of Infections A and B

Infection with virus A usually results in epidemics or outbreaks, but infection with virus B is more frequently sporadic or subclinical. Nevertheless sporadic and subclinical infection with virus A can occur, and localized outbreaks or more widespread epidemics of B infection have been reported.

In 1945–6, B virus appears to have caused a number of small outbreaks in semi-isolated communities, particularly in younger persons (see, e.g., Dudgeon *et al*, 1946), Jackson (1946) recorded a number of outbreaks of B infection in service units where West Indians were selectively attacked.

As regards the periodicity of epidemic prevalences, it has been suggested that influenza A has a cycle of 2–3 years, while that of B is from 4–6 years (Commission, 1946 *a*). Stuart-Harris (1947) doubted, however, whether experience in Great Britain completely agreed with this theory.

Coexistence of Influenza A and B

Infection with viruses A and B may occur at the same time in a single epidemic, and certain persons may suffer recurrent attacks at short intervals, due to infection with the 2 strains (Lennette *et al.*, 1941; Hare, Hamilton, and Feasby, 1943; Vilches *et al.*, 1943 *a*, Burnet, Stone, and Anderson, 1946).

Antigenic Structure of Epidemic Strains

Apart altogether from the major antigenic differences between types A and B, lesser antigenic differences have been noted between strains occurring in a single epidemic. The concept is, therefore, advanced of the possibility of a number of related strains assuming epidemic spread at the same time (Burnet and Lush, 1940 *a*, Taylor and Dreguss, 1940 *b*, Magill and Sugg, 1943, 1944, Barnes, Morgan, and Finland, 1948).

Influenza Y

Observations in many countries have shown that during an epidemic of influenza proved due to virus A or B, 30–70 per cent. of cases, although clinically typical, fail to show serological evidence of infection, and no virus can be isolated from throat washings (Martin and Fairbrother, 1939, Brown *et al.*, 1941, Lennette *et al*, 1941, Pearson *et al*, 1941, Rudd, 1941, Taylor, Petrilli, and Dreguss, 1941, Hare, Auger, and McClelland, 1942, Taylor *et al.*, 1942–3, Burnet, 1943 *c*; Hare, Hamilton, and Feasby, 1943, Hare, Stamatis, and Jackson, 1943, Stansfeld and Stuart-Harris, 1943, Vilches *et al*, 1943 *a*, Silk, Menke, and Francis, 1944, Stuart-Harris, 1945 *a*, 1947, Sigel and Davis, 1947)

The explanation of influenza Y is obscure, but there are various alternatives (see Stuart-Harris, 1945 *a*)

1. There may be a type of influenza virus differing serologically from A and B, yet undiscovered—a virus that cannot infect experimental animals readily and is noninfective for eggs

The Commission on Respiratory Diseases (1946 *c*, *d*) drew attention to what appears to be a new disease that they call "acute undifferentiated respiratory disease" or ARD Epidemiologically, ARD has features distinct from influenza, and occurs in sharply defined outbreaks in the winter among new recruits. It is probably due to a transmissible agent (see Ch. LII)

2. These cases may be caused by the low-grade "basic" virus of Andrewes (see below).

3. These cases may be infected with the prevalent A or B strains but fail to

develop antibody response There is no reason, however, why such cases should not yield virus from washings.

Incidence of Attack

A usual attack rate in the more severe epidemics has been between 10 and 20 per cent. of the populace. Thus, Huber (1933) studied 5 epidemics in soldiers in New York, occurring in 1922, 1926, 1928, March 1932, and December 1932. He found that in the 1st epidemic 9 5 per cent. of the population was affected, but 20 per cent in the 5th Underwood (1934), studying over 17,000 persons in Leeds (England), found an attack rate of approximately 16 per cent In the 1936–7 epidemic in London, Blood (1937) reported an attack rate of 19 1 per cent in factory workers.

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Virus may be spread by direct contact, as it can survive on the skin for several minutes (see p. 628).

There are a number of observations pointing to the spread of infection by carriers apparently healthy; thus cases of influenza have broken out in an island or port following the arrival of a ship (see Cheverton, 1937) Høygaard (1939) described a serious outbreak affecting Eskimos 2 months after the annual visit of a trading ship Similar observations have been made in Newfoundland (Smyth, 1947) In lonely islands and other areas where communication is very limited, the infection may be spread by farm hands and others who move about from community to community (see Cheverton, 1937)

In Alaska, Pettit, Mudd, and Pepper (1936) could definitely trace the spread to lines of air travel Smillie (1930) reported an interesting outbreak in Labrador in 1928 The affected community was absolutely isolated from the outside world except through the visit of one member to a trading station. This member returned

home with influenza and soon the disease began to spread, ever-widening circles of contacts being involved Although it is often supposed that infection may be disseminated in public vehicles, the statistics of Blood (1937) in the London epidemic of 1936 did not support this contention. Greenwood (1944) has suggested that the present custom of working in cities and traveling to the periphery at night would favor the emergence of an infection such as influenza

It is often possible to trace the spread of the disease from town to town in a given country. Thus Gover (1936) has studied the direction of spread of 11 American epidemics since 1919 (see also Collins, 1930).

Influence of environment. Blood (1937) suggested that the factory and industrial worker is the first to be attacked in the community, and that there is a higher incidence of influenza in this class of worker. Earlier, Underwood (1934), however, had not found the same state of affairs, reporting that factory workers are less susceptible than clerks and outdoor workers.

Weather conditions Influenza epidemics occur mainly in the winter, but it is uncertain exactly how cold and wet act. It may be that damage to the ciliated nasal mucosa is of importance (Burnet and Clark, 1942). Underwood (1934) found that an increased incidence of influenza followed a drop in the temperature, particularly when there was associated fog.

Cheverton (1937) made the following interesting observations in the Falkland Islands (*a*) 48 hours before an outbreak there was a drop in barometric pressure (*b*) This was associated with a drop in the maximum temperature, and 24 hours later by a rise (*c*) In addition, 48 hours before onset there was a cold, damp, southeasterly wind, an increased rainfall, and little sun. (*d*) Also 72 hours before an outbreak he found a drop in the minimum temperature

The Origin of Influenza Epidemics

Influenza epidemics occur regularly in many parts of the world, and as seen above, virus A has an approximate 2–3 year cycle, whereas that of virus B is about twice as long. What mechanisms determine why an outbreak should occur, and how is the infection kept smoldering between epidemics? This subject has received attention from Andrewes (1942 *c*, 1946), and Stuart-Harris (1945 *a*), who draw attention to various possibilities

1 Influenza may tend to travel from country to country, so that an epidemic is usually imported Certainly there have been a number of reports, mentioned above, of influenza being imported into isolated communities, but as a rule influenza A tends to occur in Britain, Europe, and N America more or less simultaneously, without much evidence of importation of infection In 1945, however, there was evidence that influenza B started in June in Hawaii and spread east, giving rise to outbreaks in the Caribbean, N and S America, and particularly in Texas, there was then a slower spread through the U S. A Holland and Belgium were involved in December, and Britain in January Australia was involved in November The Australian and British strains were closely related antigenically, suggesting spread from a common focus (see also Dudgeon *et al*, 1946)

2 A second possibility is that influenza viruses are always present in the community, causing sporadic and subclinical infections in interepidemic periods There is no doubt that such subclinical infections do occur, and it would appear that this explanation is a likely one It is possible that the endemic and imported infections may at times be superimposed

3. It may be that influenza virus strains capable of causing widespread epidemics transfer to an animal host between epidemics The susceptibility of the pig is well known, and, as mentioned elsewhere, it has been suggested that the 1918 pandemic strain attacked this animal, producing swine influenza. Influenza virus can infect various rodents, and some such host may be postulated This theory is speculative, and there is little evidence that any such transfer occurs at the present day, even if

it did 30 years ago. It is difficult to see what factors would activate the virus in the intermediate host, and how the virus would then resume its human spread.

4. Influenza virus may exist in various phases, or grades, characterized by differing degrees of virulence for man and animals (Andrewes, 1942 *c*). Thus, Grade 1 virus is the basic, nonvirulent form. Such a virus may exist in interepidemic periods and be stimulated into increased activity by exogenous factors. In this connection the activation of swine influenza virus by injections of *H. suis* should be recalled A factor tending to increase the virulence of such a basic virus would be the existence of catarrh, facilitating exchange of upper respiratory flora. A virus so increased in virulence, Grade 2, could cause a mild form of influenza such as influenza Y. This type of virus would not be sufficiently virulent to be transmissible to experimental animals, accounting for the negative results in influenza Y. A virus of slightly greater virulence, Grade 3, would be transmissible with some difficulty to ferrets, and probably would not infect mice Grade 4 virus, still more virulent, would infect ferrets fairly readily, but mice with more difficulty. It is known that viruses with the properties of Grade 3 and 4 do occur in years of non-epidemic prevalence. Grade 5 virus would be readily transmitted to ferrets and mice, and would be responsible for the typical widespread epidemics occurring every 2–3 years Grades 6 and 7, of highest virulence, would be pandemic strains, 6 attacking chiefly young adults, and 7 being highly pneumotropic.

According to this attractive theory, outbreaks of influenza arise largely as the result of a mutation of the influenza virus to a greater degree of virulence.

5. So far we have been concerned chiefly with the properties of the parasite as an explanation of the origin of epidemics It is probable, though, that varying degrees of immunity of the human host also play some part in determining the onset of an epidemic. Burnet and Clark (1942) attach some importance to the antibody

1941) Stuart-Harris *et al.* (1938) found much higher rates in naval recruits as compared with older men Investigating the incidence in isolated Yorkshire dales, Pickles, Burnet, and McArthur (1948) suggested that there was evidence for a group of immunity lasting for 4 years and diminishing the attack rate.

In conclusion it would seem that the origin of influenza epidemics depends on a variety of circumstances, partly an increased virulence of a strain previously latent, partly on facilities for its rapid transfer, and partly on the degree of immunity of the population.

Preventive Measures

Needless to say, during epidemics all crowded places such as cinemas and theaters should be avoided. Children with influenza should be excluded from school, closure of schools may be of service in localities where the children live in widely separated homes, and will avoid contact one with another if they do not

demic prevalence, but this

, disinfection of the atmosphere of public buildings and vehicles by agents such as propylene glycol, or triethylene glycol (Robertson *et al.*, 1941, 1943) A mask soaked in iodine (which has a powerful action on the virus) may also prove of value (Stone and Burnet, 1945)

LABORATORY DIAGNOSIS

Methods for the laboratory diagnosis of influenza have been greatly improved of recent years, and a wide variety of techniques is now available

A. Isolation of Virus

Preparation of inocula.

Every attempt should be made to isolate the virus from patients, partly to establish the diagnosis of influenza, and partly so that the strain can be analyzed antigenically.

Virus is sought in garglings or throat washings, the patient being instructed to gargle about 10 c.c. of broth or saline. This can be mixed with 5 c.c. of broth, and roughly clarified by paper filtration. Such "raw" material can be inoculated intranasally in ferrets and other animals (see below); or a Gradocol membrane (APD 0.8 μ) filtrate can be prepared (Burnet and Foley, 1941 *a*). Hoyle (1944) recommends filtration through sintered glass filters (maximum PD 1.5 μ).

Filtrates can also be prepared from lung suspensions. Himmelweit (1943) rec- [illegible] issue suspensions at 3000 r.p.m. for 30 minutes, [illegible] 3 superimposed Gradocol membranes arranged [illegible] 1.5, 1.05 and 0.7 μ respectively). Alternatively a Berkefeld filtrate can be used, preferably obtained after preliminary clarification (Parker *et al.*, 1946).

Certain workers have attempted to concentrate any virus present in throat washings. Thus washings can be treated with red cells to adsorb virus, and after 8–10 minutes zephiran (1/20,000) is added to keep down contaminants. After 25 minutes the cells are spun down, and then resuspended in 1 c.c. of saline which is available for inoculation in eggs (*U.S. Nav. Med. Lab.*, 1946).

Alternatively the garglings are centrifuged for ½ hour at 12,000 r.p.m. to throw down organisms. Human O cells are added to the supernatant to a final concentration of 1 per cent. Finally the red cells plus adsorbed virus are spun down and resuspended in 1 c.c. saline which can be injected in eggs (*U.S. Nav. Med. Lab.*, 1946).

More recently, antibiotics have been added to new material prior to inoculation.

If it is not convenient to examine throat washings immediately, they can be stored for several months in the cold, preferably at −76° C (Francis *et al.*, 1937 *a*, Hirst, 1942 *b*, Taylor and Parodi, 1942).

Inoculation of ferrets and hamsters.

Virus was originally isolated by the Hampstead and other workers by nasal instillation of ferrets, and this method should still be tried whenever possible (for technique and clinical features see section on ferret, below). Ferrets should be inoculated with clarified garglings or Gradocol filtrates. It is usual to pass suspensions of turbinates or lungs 3–5 times before regarding a result as negative.

As emphasized by Andrewes and mentioned above, virus strains vary greatly in their infectivity for ferrets. Typical epidemic strains of virus A usually can be established without much difficulty, but strains recovered in interepidemic periods may be difficult to establish, even by "blind" passage (Burnet and Lush, 1940 *a*, Lennette *et al.*, 1941, Taylor and Dreguss, 1941, Andrewes, 1942 *c*, Andrewes and Glover, 1944). B strains seem to be difficult, and often impossible, to adapt to ferrets (Eaton and Beck, 1941, Taylor *et al.*, 1942–3, Dudgeon *et al.*, 1946; Hirst, 1947 *b*).

Hamsters can largely replace ferrets, and after passage, strains can be passed to mice (Taylor, 1940, Lennette *et al.*, 1941, Taylor and Parodi, 1942, Taylor *et al.*, 1942–3, Eaton *et al.*, 1945).

Instead of killing ferrets or hamsters, and passing their tissues, the animals may be bled 2 weeks after intranasal inoculation of washings, and their sera examined by any of the recognized methods for neutralizing or CF antibodies (Magill, 1940, Hirst, 1942 *b*, 1945, Taylor and Parodi, 1942, Parodi, Pennimpede, and Vilches, 1944, Eaton *et al.*, 1945).

Inoculation of mice.

As a general rule, it is not possible to pass influenza virus direct from man to mouse by nasal instillation of garglings (Andrewes, Laidlaw, and Smith, 1935, Burnet and Lush, 1940 *a*, Mulder, 1940 *a*, Taylor and Dreguss, 1941). Some workers have, however, achieved success when lungs are passed in series (e.g., Sulkin, Smith, and Douglass, 1941; Sulkin, Bredeck, and Douglass, 1942; Forster and Shaughnessy, 1946, and see p. 601).

The ease of adaptation of ferret-passaged material to mice varies considerably, and strains in Andrewes' low grades may not infect mice at all Generally after a few ferret passages virus can be passed fairly readily to mice (Burnet and Lush, 1940 *a*, Magill and Sugg, 1943; Andrewes and Glover, 1944) Japanese workers have claimed the isolation of strains from centrifuged washings in mice blockaded with India ink (Tsurumi, Ogasawara, and Fujil, 1941). B strains isolated in eggs by Dudgeon *et al.* (1946) could not be adapted to mice (see also Burnet and Stone, 1946, Hirst, 1947 *b*).

Instead of attempting the isolation of virus by passing suspensions of mouse lungs, some of the animals inoculated intranasally with paper filtrates of garglings can be bled after 14 days and their sera tested for antibody, the remainder of the batch can be tested for the development of the immune state by the inoculation of a stock strain (Burnet and Foley, 1941 *a*).

Other animals.

According to Weir (1944) cane and bush rats can also be used

Inoculation of eggs.

The fertile egg has been widely used in the isolation of influenza viruses from raw washings, filtrates, or washings treated with antibiotics and antiseptics (For details of the changes produced in the fertile egg see the appropriate section below) In general, evidence of infection is obtained by the presence of chick cell agglutination with the egg fluids, or by the pathological changes present in the embryo

Eggs may be inoculated with filtrates in the yolk sac (Beveridge, Burnet, and Williams, 1944), or on the chorio-allantoic membrane (Chapman and Hyde, 1940), but these routes are not much used

Allantoic inoculation is more widely employed

(*a*) Raw washings (0 2 c c.) can be inoculated without any treatment, influenza virus will develop in a proportion of the eggs even if bacterial contaminants grow also (Thigpen and Crowley, 1943; Adams, Thigpen, and Rickard, 1944, Crowley, Thigpen, and Rickard, 1944, Rickard, Thigpen, and Crowley, 1944, 1945, Eaton *et al* 1945, Hirst, 1945, *U.S. Nav Med. Lab.*, 1946).

(*b*) Such raw washings can be treated with penicillin (*Hirst, 1945, Rose, Millow, and O'Neill*, [illegible] 1946), penicillin and sulfonamides [illegible] Anderson, 1946), or streptomycin [illegible] Ienle *et al.*, 1946 *a*) or Zephiran, 1/20,000 (*U.S Nav Med Lab*, 1942 *a*) can also be added to the raw washings, which are inoculated after 15–20 minutes contact

(*d*) Gradocol filtrates can be used.

After inoculation by any of the above methods, allantoic fluid is tested for virus by the chick cell agglutination technique (see Ch LXI). If the result is negative, the fluid should be passed through 3–5 more series of eggs Hirst (1947 *b*) found that B strains were not easy to adapt to the allantoic cavity.

Amniotic inoculation is probably more sensitive than allantoic inoculation, and as shown by Burnet (1940 *a*, *b*) is an excellent method of isolating virus As some

embryos fail to show evidence of infection, 6 should be inoculated with each sample.

(*a*) Raw washings treated with penicillin (Hirst, 1945, Francis, Salk, and Brace, 1946), or penicillin and sulfonamides (Burnet and Stone, 1945 *c*, Burnet, Stone, and Anderson, 1946, Dudgeon *et al.*, 1946) may be inoculated (0.1 c c.). McKee and Hale (1947) recommend 500 units of penicillin and 1000 units of streptomycin per c c. These are perhaps the most satisfactory methods of laboratory diagnosis in influenza.

Burnet and Stone (1945 *c*) recommend the following technique: The patient gargles 10 c c of saline for one minute, 5 c.c. of broth is then added, and enough penicillin to make a final concentration of 100 Oxford units per c.c. This material is filtered through sterile filter paper to remove gross particles Amniotic inoculation with 0 1 to 0.25 c c. of washings is then performed in 13–14 day embryos, 0.1 c c. of 5 per cent. sodium sulfadiazine being first inoculated in the same cavity. Embryos dying before 48 hours are discarded.

(*b*) Chemicals, as mentioned above, may be used.

(*c*) Most work has been carried out with Gradocol (APD 700–750 mμ), or similar bacteria-free filtrates (Burnet and Foley, 1940, 1941 *a;* Rudd, 1941, Stuart-Harris, 1941; Burnet *et al*, 1942, Hirst, 1942 *b*, 1943 *b*, 1945; Burnet and Bull, 1943; Burnet, Beveridge, and Bull, 1944; Beveridge, Burnet, and Williams, 1944, Hoyle, 1944; Eaton *et al.*, 1945; *U.S. Nav. Med. Lab.*, 1946).

The presence of infection is shown by chick cell agglutination given by the amniotic or allantoic fluid, and by the characteristic tracheal exudate and lung changes (see p. 620). If the first passage proves negative, passages should be continued with allantoic fluid, amniotic fluid, or ground lung or trachea (10 per cent. suspensions). Virus B may be less easy to isolate than virus A, but sometimes seems to grow better in 8–10 day embryos, a number of Australian B strains in 1945 was however, isolated in 13–day eggs (Beveridge and Burnet, 1946) Hirst (1947 *b*) found that it was necessary to passage to isolate B strains It must be remembered that normal amniotic fluid after about the 13th day causes agglutination of various red cells, but not of chick cells, the titer, however, is not above 32 (*Commission*, 1946 *b*). Some normal allantoic fluids of high protein content give an atypical agglutination of guinea-pig cells, but not beyond 1/10 (Beveridge and Burnet, 1946).

Inoculation of tissue cultures.

It has been claimed that virus can be isolated by inoculation of tissue cultures (see p 616)

B. Serological Tests

A sample of patient's serum should be obtained during the acute phase of illness, and a further sample 2 weeks later Both samples are then tested at the same time for a rise in virus neutralizing, agglutination inhibiting, or complement fixing antibodies (see Ch LXIII) A twofold, and certainly a fourfold increase in antibody titer indicates infection It should be realized, however, that a number of cases of undoubted influenzal infection, especially those with a high initial titer, will fail to show any increase in convalescence

C. Blood Picture

With regard to the blood picture, leukopenia was usually found in pandemic, and may occur in epidemic influenza (Burden, 1933, Reyersbach, Lenert, and Kuttner, 1941), *especially when secondary infection depresses hemopoiesis* (Parker *et al.*, 1946) Usually, however, the count is relatively normal (Stuart-Harris *et al*, 1938, Stuart-Harris, 1945 *a*, Adams, Thigpen, and Rickard, 1944) It appears that uncomplicated influenza may be accompanied by a leukocytosis (Scadding, 1937, Parker *et al.*, 1946).

CHAPTER LVI

ANTIGENIC CHARACTERISTICS OF INFLUENZA VIRUS

ISOLATION AND STRAINS OF INFLUENZA VIRUS

Early Work

THE VIRUS now known as Type A was originally isolated by the National Institute

36–7 (Smith,

aidlaw, 1935;

isolated this

ated in Mel-

France (Du-

with A and B virus according to the infection present. We refer to viruses A or B as "types," and individual strains belonging to either type as "strains"

Attempted Isolation in Interepidemic Periods

As explained above, the clinical features of influenza, although characteristic, do not always enable a differentiation to be made from other infections of the respiratory tract Virus is not usually recoverable from cases with influenzal symptoms

1935, Fair-

umotropic"

Strains of Virus

The following are the best known strains of virus A· WS, PR8, Philadelphia, Leningrad, Melbourne, Christie, Talmey, Weiss, Bel, and CC

The best known strains of virus B are Lee, TM (Magill and Tyndall, 1941), Montgomery (Eaton and Beck, 1941), Paddington (Himmelweit, 1943), Bon (Burnet, Beveridge, and Bull, 1944). A number of other B strains has also been studied (Eaton *et al*, 1942, Gordon, 1942, Francis, Salk, and Brace, 1946, Burnet, McCrea, and Stone, 1946, Burnet and Stone, 1946, Dudgeon *et al*, 1946, Francis, 1947 *a*).

ANTIGENIC STRUCTURE OF INFLUENZA VIRUS

The antigenic structure of influenza virus is complex, and has been much studied of recent years. Most of the information has been obtained by differential centrifugation, serological tests (especially complement fixation and the chick cell agglutination test), and electron microscopy. These subjects will all be dealt with later in appropriate sections, but it has been thought desirable to bring together in this section all observations relating to the structure of influenza virus.

[1] References are appended at the conclusion of Ch LXIII, p. 671 et seq

First suggested by the work of Smith (1936), Hoyle and Fairbrother demonstrated conclusively that influenza virus was of complex structure, using mouse lung suspensions (Hoyle and Fairbrother, 1937 *b*, *c*, Fairbrother and Hoyle, 1937 *b*, Fairbrother, 1938). By differential centrifugation they separated 2 fractions, the elementary body (EB) and the soluble substance (SS). They were able to show that these 2 substances have very different properties: The EB's are associated with infectivity. They stimulate resistance to infection in mice. Virus neutralizing, and to a lesser degree complement fixing, antibodies develop after their injection. Even when EB's are rendered noninfective by heating at 57° C., mice and ferrets can still be immunized.

The soluble substance they found to be noninfective. On injection into mice it fails to stimulate resistance to infection, virus neutralizing antibodies do not develop, and complement fixing antibodies only to a slight extent. The soluble substance acts as a powerful antigen in complement fixation tests, and is found abundantly in the supernatant fluid of saline extracts of lung.

These observations have since been confirmed and extended by other workers, many of whom have used infected allantoic fluid as source of virus, rather than lung suspension (Eaton, 1940 *a*, Lennette and Horsfall, 1940, 1941, Chambers and Henle, 1943, Chambers *et al.*, 1943, Friedewald, 1943; Friedewald and Pickels, 1944, Henle and Wiener, 1944, Henle *et al*, 1944, Lauffer and Miller, 1944 *b*; Stanley, 1944 *a*, *b*, Hoyle, 1945; Wiener, Henle, and Henle, 1946, see also section on centrifugation below)

When preparations containing influenza virus A or B are centrifuged, the elementary bodies are thrown down at a lower speed than the soluble substance (SS). The EB's have a sedimentation constant of about 600 S, and that of the soluble substance is about 30 S, there is also a 3rd component known as < 30 S (Henle and Wiener, 1944, Henle *et al*, 1944; Stanley, 1944 *b*, Wiener, Henle, and Henle, 1946, see also section on centrifugation below). The properties of these 3 components are as follows

Elementary Bodies, or 600 S Component

1. The size of these particles, calculated from centrifugation data, is 80–120 mμ.
2. They are associated with infectivity.
3. They cause red cell agglutination.
4. They produce toxic lesions in mice.
5. They produce the interference phenomenon
6. They can immunize animals, which develop virus-neutralizing and other antibodies and resist infection

Antibodies to 600 S develop in man after both infection and vaccination (Wiener, Henle, and Henle, 1946) Absorption of antisera with 600 S removes their ability to react with 600 S in the CFT, capacity to inhibit chick cell agglutination, and to neutralize mouse infectivity (Wiener, Henle, and Henle, 1946)

7. EB's act as an antigen in complement fixation
8. EB's cannot be entirely freed from SS by washing, and it appears that they are partly composed of SS (Lennette and Horsfall, 1940, Hoyle, 1945). Experiments with absorbed antisera have shown that 30 S is part of 600 S (Wiener, Henle, and Henle, 1946)
9. SS is released from 600 S by partial disintegration (Henle and Wiener, 1944), and sonic vibration (Wiener, Henle, and Henle, 1946) The amount of SS in the elementary bodies varies with different strains (Stanley, 1944 *b*)
10. EB's probably contain 3 or 4 antigenic components as well as SS (Hoyle, 1945). Some of these components are shared by related strains, but others may be specific (Friedewald, 1943, Henle and Wiener, 1944, Wiener, Henle, and Henle, 1946) Antigenic differences between strains of one type are thought to be due to quantitative rather than qualitative variations in antigenic components

11. The complement fixing property of the elementary body can be adsorbed by fowl cells and eluted therefrom, and cannot be separated from the virus by repeated washings or adsorptions (Friedewald, 1943).

Soluble Substance or 30 S Component

Soluble substance is found most abundantly in the supernatant fluid of mouse lung suspensions, and can also be obtained b [illegible] fluid It can be found in saline extracts of [illegible] Fairbrother, 1937 *c*), but very little is foun [illegible] and Horsfall, 1941) *The properties of soluble substance are as follows* [illegible]

1. The component is thrown down by a speed of 30,000 r.p.m., and the size of the particles is about 10 mμ (Lennette and Horsfall, 1940, Henle and Wiener, 1944; Stanley, 1944 *b*).
2. This component is not associated with infectivity, apart from occasional contamination with 600 S particles
3. It does not agglutinate red cells, and is not adsorbed by them
4. It does not give toxic lesions in mice
5. It does not produce the interference phenomenon
6. Antibodies to 30 S develop in man regularly only after infection, not after vaccination (Wiener, Henle, and Henle, 1946)
7. SS acts as a powerful antigen in the complement fixation test.
8. The soluble substances of various strains of virus A appear to be identical and distinct from those of virus B, SS appears to be a single antigen (Lennette and Horsfall, 1941; Friedewald, 1943, Henle and Wiener, 1944; Hoyle, 1945, Wiener, Henle, and Henle, 1946).

The SS of swine virus resembles, but is probably not identical with that of virus A (Lennette and Horsfall, 1941).

< 30 S Component

This component behaves serologically like 30 S, and remains suspended even after repeated centrifugation at 30,000 r.p.m It gives complement fixation, and appears to consist of a single antigen (Henle and Wiener, 1944).

"Normal" Components in the Structure of Influenza Virus

As so much work has been performed with preparations of influenza virus from infected allantoic fluids, it is necessary to inquire whether normal allantoic antigen may not enter into the structure of the virus. Such is, in fact, the case Thus Knight (1944 *c*) isolated a macromolecular "protein" material by high speed centrifugation from normal allantoic fluid The yield was maximal after 14 days incubation at 39° C (about 0.02 mgm per c.c of fluid) The properties of this material were

1. It contained protein, carbohydrate, lipoid, and *in general the composition* resembled that of purified PR8 virus
2. The isoelectric point was pH 2.3, whereas that of virus is usually *around 5.*
3. The normal material had a variety of sedimentation constants *depending on* the concentration and viscosity of the fluid The sedimentation of the *major component* was 170 S
4. The diameter of the predominant particles, *shown by electron micrographs,* *was about 40 mμ, whereas influenza* virus measures about 100 *mμ.*
5. The normal protein did not agglutinate *chick red cells and was not absorbed* by them
6. The normal protein was a good antigen, and *antiserum could be prepared in*

rabbits. Precipitin tests showed that preparations of influenza virus, purified by centrifugation of infected allantoic fluid, contained normal protein material. This normal material could not be removed by repeated freezing and thawing of allantoic fluid and repeated adsorption of virus on to red cells.

7. From 2 virus strains (F12 and Lee) a small component, closely related to or identical with normal protein, was separated by centrifugation.

8. Antisera to the purified normal material inhibited red cell agglutination by A and B strains at serum dilutions of 600–700, whereas antisera to purified PR8 inhibited at 112,000. Antiserum to normal protein did not show any neutralizing effect in chick embryos or mice.

Knight (1946 *b*) later extended this work, and confirmed that the best preparations of virus from allantoic fluid now available are still grossly contaminated with nonviral material. In these later studies he obtained purified preparations of sedimentable normal allantoic protein. Purified virus preparations were obtained by adsorption to and elution from chick red cells, followed by centrifugation. Particles from normal and infected mouse lungs were also used. Antisera were prepared to these various normal and infective particles in rabbits, and precipitin tests performed, with the following results:

1 With antiserum to normal allantoic protein, heavy precipitates were obtained with highly purified PR8 and Lee strains from allantoic fluid. No precipitate was, however, obtained with PR8 particles from mouse lungs. Highly purified preparations of PR8 and Lee viruses from allantoic fluid contained about 20 per cent. and 30 per cent. respectively of normal protein

2. With antiserum to purified PR8 from allantoic fluid, precipitates were yielded by homologous antigen, normal allantoic protein, Lee virus from allantoic fluid, and PR8 from mouse lung. Normal lung particles gave no significant precipitate

3. The antibodies in PR8 antisera responsible for cross precipitation with Lee virus did not neutralize the infectivity of Lee virus.

4 About 96 per cent. of the infectivity of the most highly purified allantoic virus preparations could be precipitated by antiserum to normal allantoic protein

5 After a single chick embryo passage, mouse lung virus became indistinguishable from egg-adapted virus in precipitin tests, showing that it acquired an antigenic component from the new host.

In later work (1947 *a*) on the chemical structure of PR8 and Lee strains, he suggested tentatively that glucosamine might be derived entirely from the host, if this assumption was correct, a revised estimate of the amount of normal host material present in PR8 and Lee particles would be 59 per cent. and 78 per cent. respectively

This important work of Knight shows that host antigen–mouse or chick, for example–is incorporated in the intimate structure of the virus particles Knight suggests that there is a common antigenic component for a given strain of virus present in all hosts In addition to this intrinsic virus fraction, each host yields a component of its own Antibodies to the viral component neutralize virus infectivity, and inhibit agglutination of chick red cells by virus, irrespective of the source of the preparation of virus acted upon

Wiener, Henle, and Henle (1946) found that Knight's normal allantoic protein did not react in fixation tests with human convalescent sera

Francis' "Complex Antigen"

Francis (1947 *a*), who has studied the effect of heat on the hemagglutinating capacity of influenza virus B strains in agglutination inhibition tests (see p 637), has postulated the presence of a complex antigen consisting of a heat-stable component and a heat-labile component The precise relationship of these observations to those already mentioned is not at present clear.

ANTIGENIC DIFFERENTIATION OF TYPES AND STRAINS OF INFLUENZA VIRUS

Methods

Preparations of the virus to be analyzed can be obtained from mouse lung, if necessary, EB's and SS can be obtained by suitable differential centrifugation. It is often more convenient to make use of the susceptibility of the allantoic and amniotic cavity to yield a generous sample of virus-containing fluid

As regards antisera, rabbit sera have been much used, but are probably not so specific as ferret sera. Serum is obtained from rabbits after 1 or 2 intraperitoneal injections of living virus, if more virus is given, the antibody response tends to be too "wide." Ferret sera obtained about 2 weeks after experimental infection are highly specific. Human sera are not usually sufficiently specific for this type of work. Burnet and Stone (1946), however, in work with B strains, used the sera of adolescents, they applied a correction to the crude antibody titers to allow for the differing avidities and varying antibody content. The sera of fowls have been recommended, being removed 15 days after an intraperitoneal inoculation of virus (Hudson, Sigel, and Markham, 1943). Mouse sera have also been employed (Eaton, 1941)

1 As regards the tests used, the unknown strain can be exposed to the action of sera prepared against known strains, and the mixtures tested for infectivity by mouse inoculation, or inoculation in eggs or tissue cultures The known strain is tested likewise, and an estimate of their affinity can be expressed on a percentage basis

2 Complement fixation tests can also be carried out, preferably separately with antigens prepared from EB's and SS of the unknown strain, and appropriate known antisera

3. Passive protection experiments with antisera to known strains have also been used, the unknown strain being inoculated nasally in a susceptible animal.

4. The precipitin test was used in Knight's work (see above), but is of little use in differentiating between strains. Tests involving absorption of antibodies can be used in accurate work (Ch. XI).

5. It is now customary to use the chick cell agglutination test in analyzing the antigenic structure of an unknown strain (Hirst, 1942 *b*, *c*, 1943 *b*, see Burnet and Clark, 1942). Thus, the unknown strain is mixed with known antiserum in the presence of chick cells If agglutination occurs, then the unknown strain differs from the strain used to prepare the serum; if there is no agglutination, an antigenic similarity can be inferred. If the known immune serum is titrated in the presence of the unknown strain, and a parallel test set up with the known serum and known strain, it is possible to assess how nearly the unknown strain resembles the known. In accurate work it may be necessary not only to investigate the antigenic structure of the EB's by the chick cell test, but also of the SS by complement fixation It may also be necessary to prepare antiserum to the EB's and use this serum in tests with known strains. Hirst's method has the advantage that it is not necessary to use mouse-adapted strains of virus, it can, therefore, be carried out quickly with antigen derived from allantoic fluid in the first or early passage from the human material

6 Another type of test involves active immunization of mice with known strains, and subsequent challenging with the unknown, *vice versa*, the unknown can be used to immunize, and the known to challenge Antigenic differences be-

[illegible] a single previous

strain of influenza

Inoculate the amniotic cavities of 4 to 6 12-day chick embryos with a Gradocol filtrate of throat washings.

Inoculate mice intranasally with amniotic fluid from those embryos showing typical changes.

Bleed some of these mice, and test for the amount and type of influenzal antibody against known strains. Test the resistance of the remainder by the inoculation of a standard adapted mouse strain intranasally.

Virus A

For a time it was thought that all strains of influenza virus were antigenically similar. For instance, Francis (1934–5) found that the sera of ferrets convalescent from infection with either his PR8 or Phila strains neutralized both the homologous and heterologous strains. Andrewes, Laidlaw, and Smith (1935) isolated 6 new strains and found them serologically identical Brightman (1936) found that his New Haven strain was neutralized by antisera to PR8 and Phila strains. Stokes *et al.* (1937 *b*) found that 3 strains isolated in Philadelphia were neutralized by antiserum to the PR8 strain. French strains studied by Dujarric de la Rivière and Chevé (1937 *b*) all appeared to be identical serologically with the WS and PR8 strains.

Later results, however, conclusively showed that antigenic differences do exist between certain strains. Thus, Magill and Francis (1936–7) found a difference between their PR8 and Philadelphia strains by the use of rabbit antisera. Burnet (1937 *b*), who studied the question in detail, was able to divide strains into 3 groups on the basis of antigenic differences (*a*) the New World type consisting of Melbourne, *Phila*, and *PR8* strains, (*b*) the Old World type consisting of WS, BH, Moscow and Leningrad strains; (*c*) swine influenza virus. In grouping these strains he used neutralization tests with convalescent ferret serum in mice and on the chorio-allantois, active immunization of mice and ferrets, and antibody-absorption.

Stuart-Harris *et al* (1938) found that strains isolated in 1936–7 were different antigenically from those isolated in earlier epidemics. Francis (1938) carried out another study of this problem, using serum neutralization tests and active immunization of animals He found that the majority of 1936–7 strains was closely related one to another, but differed from the earlier strains. Further, he made the important observation that strains of different antigenic constitution might occur in the same epidemic.

Certain papers established beyond all question the antigenic heterogeneity of influenza virus A strains (Magill and Francis, 1938 *a*, Francis and Magill, 1938 *a*, Smith and Andrewes, 1938, Andrewes, 1939) In this work immune sera were used to detect antigenic differences, and so were experiments involving active immunization and serum protection of mice In addition, it was possible to show that serum antibody could be demonstrated by its effect in preventing the growth of virus in tissue culture (Magill and Francis) The most important findings of these groups of workers may be summarized as follows

1. Twenty-eight different human strains were examined by Smith and Andrewes, and 23 by Magill and Francis

2. The strains that resemble each other most closely are those from the same epidemic, different serological types may be isolated from the same epidemic (Magill and Francis)

3. Variations between strains cannot be accounted for by the effect of animal or tissue culture passage (Magill and Francis)

4. There are numerous antigenic components shared by influenza strains, and these may be considered as arranged in the form of a mosaic, the components are present in different proportions in different strains, all the components are not present in every strain (Magill and Francis, Smith and Andrewes)

5 Four components are of major importance, strains may be classified, on the

basis of these components, into 3 groups of highly specific, relatively nonspecific, and intermediate strains (Smith and Andrewes).

Other more recent observations on the antigenic differentiation of virus A strains may be quoted Thus, Hungarian strains were found to differ considerably from PR8 and WS, and to fall into a group of their own (Taylor, Dreguss, and de Ritis, 1940, Taylor and Dreguss, 1941)

Hirst (1943 *b*) analyzed 18 A strains isolated in the 1940–1 U.S epidemic, 16 appeared to belong to one group while 2 were distinct.

Friedewald (1944 *b*) studied the following strains of virus A by antibody absorption tests PR8, Christie, Talmey, WS, and the swine strain He found that PR8 had the broadest range of specific antigenic components, the other strains being more specific.

Sugg and Magill (1946) detected differences between the CC and WS strains by challenge tests in actively immunized ferrets

With regard to the effect of passage on antigenic structure, it has been shown that specific strains may change on mouse passage to less specific intermediate forms (Burnet and Lush, 1940 *a*) An antigenic difference could be made out between Melbourne Egg virus and its substrain adapted to mouse passage (Burnet and Bull, 1944). It has been found that there is an antigenic difference between a line of PR8 virus passed continually in the mouse, and another line of PR8 passed in tissue cultures and eggs (Francis, 1947 *c*)

The main observations in this connection concern the O–D change that occurs in virus on egg passage, and which is fully discussed in a separate section below.

Our present knowledge of the structure of influenza virus A enables us to understand more clearly the position in regard to antigenic differentiation between strains From what has been said above, it will appear that all strains are composed of elementary bodies and soluble substance The elementary bodies contain a number of antigenic components that may be shared by strains of the same type Differences between strains of the same type of virus are largely due to quantitative differences in antigenic components Differences between Type A and B ...
due to qualitative d
a proportion of SS
distinct from the SS of virus B Finally, all EBs contain from 20–30 per cent of antigenic material derived from the animal host or chick, at least

Virus B

Strain differences were already well recognized when Francis (1940 *a, b*) reported that he had isolated a virus (Lee) of the influenza group so distinctive anti-

those of virus A The SS of B strains is also distinct from that of A strains Both B and A strains contain a definite proportion of antigenic material derived from the host, as shown by Knight, which may account for an apparent antigenic relationship between the 2 strains

Strain differences occur with virus B just as with virus A (Gordon, 1942, Burnet, Beveridge, and Bull, 1944) Thus strains isolated in England and Australia, and in other parts of the world in 1945–6 were found to resemble each other closely and to differ from the Lee strain (Burnet and Stone, 1946, Dudgeon *et al*, 1946).

Work of Hirst (1947 *a, b*)

Recent work of Hirst suggests that the whole question of the antigenic structure of influenza viruses needs reinvestigation It appears that previous conclusions,

based on cross protection experiments with ferrets or mouse-adapted virus, regarding minor antigenic differences between strains of one type, may have been mislead[illegible], and studied antigenic structure by means of

[illegible] ntigenic structure, changed on mouse passage, so that they differed not only from one another, but from the parent also.

2. None of a number of B strains isolated in 1945 in Hawaii, Yale University, and New York differed significantly from any of the others, these strains showed distinct differences, however, from mouse-adapted Lee (B) virus

3. Confirming earlier observations made in 1940–41, Hirst found no significant antigenic differences between A strains isolated in 1943–44 from 4 different states. A slight difference was detected between A strains isolated in 1940–41, and in 1943–44.

4. He emphasizes that present conceptions of strain differences, based on mouse work, are probably inaccurate, for when egg-adapted viruses have been used, no significant antigenic differences have been revealed. It would seem logical to replace current vaccines containing mouse-adapted strains (PR8, Weiss, and Lee) by strains *as similar as possible to those current in human epidemics.*

Swine Influenza Virus

The relationship between swine and human strains has been investigated by various means Thus the sera of swine convalescent from swine influenza do not neutralize the human strain of virus A *in vitro* (Francis, 1934–5). Further, Francis and Shope (1936) reported that the sera of animals recovering from infection due to human or swine viruses were virtually specific, for little neutralization of the heterologous virus took place They found that hyperimmunization with human virus A resulted in the production of antibodies operating against the swine virus as well, whereas hyperimmunization with the swine virus only increased the human virus antibody irregularly. They concluded, therefore, that although the 2 strains were immunologically distinct they shared antigenic components Further, the common antigen was present in greater quantity in the human virus Using the complement fixation technique, Fairbrother and Hoyle (1937 *a*) also postulated a common antigen in the 2 strains A similar conclusion was arrived at by Magill and Francis (1938 *a*) and Francis and Magill (1938 *a*), using cross neutralization and cross immunization tests in mice Shope (1939), using rabbit antisera in neutralization tests, was able to distinguish human and swine strains readily

The common antigenic factor gives rise to some cross immunity when the resistance of animals recovered from the infection is tested by heterologous virus.

Thus, Shope (1937 *a*) found that swine recovered from swine influenza, or the virus infection only, are generally resistant to a latter inoculation of human influenza virus A He also found that swine recovered from infection with the virus of human influenza and *Hemophilus influenzae suis* are generally immune to swine influenza The human virus infection itself (without the bacterium) does not, however, confer immunity to swine influenza.

Investigating the nature of the immunity in those pigs that had been infected with swine virus, Shope found that it was really a refractory state The human virus was unable, in these resistant animals, to penetrate the nasal mucosa of the pig. The immunity was not due to antibodies, as swine recovered from the swine virus did not show antibodies to the human virus In distinction to this mechanism, Shope found that the mucosa of swine recovered from the human infection did allow the swine virus to pass through, the virus was, however, dealt with by other mechanisms

The antigenic structure of strains of swine influenza virus isolated in Great Britain has been investigated Glover (1941 *a*) found that they were rather different from American strains, and not so clearly distinct from human strains (see also Glover and Andrewes, 1943). Confirmatory results were obtained by Hudson, Sigel, and Markham (1943), who compared them with PR8 and WS (A), Lee (B), and Shope's swine strain They used specific inhibition of chick cell agglutination, and sera obtained from ferrets and fowls The British strains were found to be closely related to PR8 and WS and distinct from Shope's virus

RELATIONSHIP TO OTHER VIRUSES

Influenza virus has highly characteristic properties and does not resemble many other viruses, although a number share the ability to agglutinate red cells

THE O-D CHANGE

Burnet and his collaborators have drawn attention to differences in the properties of virus as freshly isolated from the host by amniotic inoculation, and the same strain after it has been passed in the laboratory. The freshly isolated virus is said to be in the O (original) phase, while the stock virus is in the D or derivative phase. D phase virus develops when strains are passaged through and adapted to eggs by the amniotic, yolk sac, or allantoic routes, unless special precautions are taken. The change that occurs is considered to be in the nature of a discontinuous

Sources of O Phase Virus

O phase virus is isolated most readily from the human case by inoculation of washings in the amniotic cavity. It has also been recovered from the nasal washings of a ferret inoculated with first passage virus (Burnet and Stone, 1945 *c*).

Strains Used

The original work was carried out with strain Bel of Type A (Burnet and Bull, 1943, Burnet and Stone, 1945 *b*) Other A strains have been isolated in the O phase (Beveridge, Burnet, and Williams, 1944; Burnet, McCrea, and Stone, 1946, Hirst, 1947 *b*).

Burnet and Bull (1944) reexamined the properties of their strain Melbourne Egg (Type A). In the early stages of chorio-allantoic passage this strain showed D hemagglutination, but in its fully developed form it resembled closely an unadapted O strain

Burnet, Beveridge, and Bull (1944) studied the properties of a strain Bon of Type B They could not demonstrate any change from the unadapted to the adapted state. Other B strains isolated by amniotic inoculation have shown no evidence of being in the O phase (Burnet, Stone, and Anderson, 1946, Dudgeon *et al.*, 1946)

Properties of the Various Phases

1 O strains in amniotic fluid do not readily agglutinate fowl cells, but human

of fowl cell ag-

) and D phases is not quite so clear cut. An almost continuous series of reactions can be observed among amniotic fluids or lung emulsions from embryos infected with O phase virus at various dilutions (Burnet and Stone, 1945 *b*). As now defined, therefore, O virus preparations show no fowl cell agglutination, or at most a titer less than 1/20 of that given by guinea-pig cells Two intermediate grades are now recognized— α and δ The F/G ratios, and the character of the deposited agglutinated red cells are as follows

O	No agglutination	F/G ratio 0/40–0/1,000
ω̄	Weak loose, rapidly disappears	1/20–1/3
δ	Streaky or insular	1/3–1
D	Continuous shield	1 or +

The agglutination reactions with red cells from various species are as follows

	O	D
Fowl	not readily	readily
Guinea-pig	equally well agglutinated	
Human	equally well agglutinated	
Pigeon	equally well agglutinated	
Ferret	very weakly agglutinated	
Mouse	very weak	readily

It must be noted that normal amniotic fluid after the 13th day contains pseudo-globulin from the albumen sac, and this can cause agglutination of guinea-pig and many other cells, but not above a titer of 32, chick cells are not agglutinated (Commission, 1946 *b*).

2. When O phase virus is inoculated in the amniotic cavity, the amount of fluid is found to be increased, but this increase does not occur with D phase, where, in fact, the volume may be reduced Multiplication of O virus is more rapid in 13–14 than 12-day embryos O virus does not usually kill the embryo in small doses within 4 days, fully adapted D strains usually kill in 2–3 days (Beveridge and Burnet, 1946).

After inoculation of O virus in the amniotic cavity, it can be recovered in this phase for the first 2–3 days, but thereafter intermediate D type virus is usually recovered After the 5th day, no typical O virus is recoverable. As a general rule, virus is recovered in the same phase in both lung and amniotic fluid

If concentrated preparations are inoculated amniotically, D phase virus results, whereas if dilute inocula are used, the amniotic fluid may give the reactions characteristic of O virus These characters may be maintained for several passages if carried out with limiting dilutions

3. O and ω̄ strains do not multiply readily in the allantoic cavity, whereas D and δ strains proliferate However, large doses of O virus will infect by the allantoic route (Burnet and Stone, 1945 *a*)

4 *O strains do not infect mice readily, but are highly infective for ferrets,* guinea-pigs, and man. Large inocula of O virus may initiate infection irregularly in mice (Burnet and Stone, 1945 *a*) D virus is not as active as O on intranasal inoculation in guinea-pigs

5 Human tears effectively inhibit [illegible] D strains, but fail to inhibit [illegible] ion by the D phase is

6. Slight ant [illegible] between the 2 phases, using ferret antisera

7 O and D viruses can be absorbed out of suspension, to a certain degree, by red cells that they agglutinate Absorption with fowl cells will not remove pure O virus from suspensions of infected embryo lung, but removes particles other than those in this phase (Burnet and Stone, 1945 *b*).

8 Certain B strains isolated by Hirst (1947 *b*) in 1945–6 had no tendency to the agglutination picture of O phase, but showed poor adaptation to mice and the allantoic cavity.

Maintenance of Virus in O Phase

Burnet and Stone (1945 *b*) outlined the following precautions to be observed in maintaining virus in the O phase during egg passage The inoculum should be prepared from embryo lung, rather than amniotic fluid, by absorption with fowl cells The fowl cells remove virus particles other than those in the O phase The

initial emulsion should show a F/G ratio of 1/10 or less, while the absorbed fluid should not agglutinate fowl cells. Dilutions are then prepared in horse serum saline, 10^{-4} to 10^{-6}, and inoculated amniotically in 13–14 day embryos It is essential to passage at limiting dilutions, for if more concentrated preparations are used, D phase virus will appear The lungs are harvested after not more than 72 hours at 35° C. O virus can be preserved in glycerolated amniotic fluid (Burnet, McCrea, and Stone, 1946).

Significance of the O-D Change

Human influenza virus A in its O phase is genetically unstable, and D phase, which is more stable, appears whenever virus has an opportunity to multiply sufficiently to provide an adequate number of replicas. In mouse and chick embryos the D type virus usually multiplies more freely and overgrows O, which requires special conditions for proliferation It is well known that as a rule 1 or 2 ferret passages are necessary before a newly isolated strain, i e, in the O phase, can be adapted to mice, i e, assume the D phase. The O form must have some specific character favoring its survival under epidemic conditions (Burnet and Bull, 1943)

O No agglutination	F/G ratio 0/40–0/1,000
ω Weak loose, rapidly disappears	1/20–1/3
δ Streaky or insular	1/3–1
D Continuous shield	1 or +

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Virus B has also been proved infective for human volunteers, the symptoms being in general similar to those recorded after inhalation of virus A, although perhaps somewhat milder, and the illness having a shorter incubation period, nausea and vomiting is not uncommon (Bull and Burnet, 1943, Burnet, 1943 *b*, Francis *et al*, 1944 *b*; Salk *et al*, 1945 *a*, Henle *et al*, 1946 *a*).

Local symptoms of sneezing and "stuffy" noses were commonly found in Bull and Burnet's series

Virus can be recovered from the nasal secretion of persons experimentally infected with virus B 48 hours later (Bull and Burnet, 1943)

By no means all persons exposed to atomized virus develop influenza, and the outcome of an experimental exposure may be considered to depend on various factors (Henle *et al*, 1946 *a*)· (1) the extent of multiplication of and invasion by the virus, (2) any toxic, as opposed to invasive, properties of the virus, (3) the presence of an allergic state due to previous "sensitizing" exposure; (4) to which may be added the state of immunity of the volunteer as evidenced by the level of antibody in the serum, or nasal secretions. Probably also some local susceptibility or resistance of the respiratory mucosa plays a part.

ANIMAL EXPERIMENTS

Influenza virus is capable of infecting a wide range of animal hosts, and in so far as the respiratory system is predominantly involved, it may be designated a "pneumotropic" virus (Waldmann, 1935).

Ferret

It was by means of the ferret that virus A was originally isolated by Smith, Andrewes, and Laidlaw (1933), and ferrets have been used extensively in later work. They are susceptible also to infection with swine strains B strains are adapted only with difficulty, if at all Certain precautions are necessary in work with ferrets, thus, they must be carefully quarantined for at least 14 days before use, as they may suffer from distemper (see Pyle, 1940).

It has been shown that ferrets may suffer from another epizootic of high fatality rate (Slanetz and Smetana, 1937, Slanetz, Smetana, and Dochez, 1936). This disease is due to a filtrable virus, and hemolytic streptococci may act as secondary invaders Spooner (1938) studied a somewhat similar disease that affected ferrets supplied in the London area in 1934–5 It presented much similarity to ferret distemper and to Slanetz and Smetana's disease Francis and Magill (1938 *b*) isolated a hitherto undescribed virus by ferret inoculation (virus of acute meningopneumonitis, see Ch LXVIII) Ferrets infected with influenza are highly infective, and readily infect others, unless rigid isolation is carried out

Method of inoculation Ferrets are usually susceptible only to intranasal instillation, administration by the intramuscular or subcutaneous routes does not cause infection. Direct intrapulmonary injection secures infection, but is not used in practice (Andrewes, Laidlaw, and Smith, 1934) The usual method of infecting ferrets is to drop about 0 5 c.c –1 5 c c. of inoculum on the nares of the animal, when held vertically Etherization may be carried out, and induces a severer infection, intraperitoneal nembutal can be used After intranasal inoculation, virus can be demon-

and Scott, 1944 *b*). They can become infected one from another over a distance of about 5 feet, probably by fine droplets, the interposition of a screen or increased ventilation prevents such cross infection (Andrewes and Glover, 1941).

Combined bacterial and virus infection. Glover (1941 *b*) found that a hemolytic

CHAPTER LVII

INFECTIVITY OF INFLUENZA VIRUS FOR MAN AND ANIMALS

HUMAN TRANSMISSION

EARLY in the course of influenza research, certain workers in London and New York developed influenza while attending to infected ferrets. The best-known case of this type is that recorded by Smith and Stuart-Harris (1936)[1] Stuart-Harris himself became infected after examining a batch of ferrets infected with passage virus, one of which sneezed in his vicinity. The whole circumstances of his attack were such as very strongly to suggest that he had become infected from this animal. The matter was finally settled when it was found that the virus isolated from Stuart-Harris possessed the properties of passage virus (actually the 196th passage), and not of fresh virus. This important observation showed that despite almost 200 passages through ferrets, the virus retained its pathogenicity for man It formed a very strong link in the chain of evidence incriminating the filtrable virus as the cause of influenza.

Further evidence for the infectivity of the virus for man was furnished by Soviet workers, Smorodintseff *et al.* (1937) inoculated volunteers with the WS (original London strain) and Leningrad strains of virus by spray. In a percentage of cases the patients developed symptoms of a mild attack of influenza.

Of more recent years, a number of experiments has established beyond doubt the pathogenicity of influenza viruses A and B for the human subject.

As regards the route of inoculation, it is generally agreed that it is not easy to infect human volunteers by simple instillation of drops of virus-containing fluid. Despite failure to cause symptoms, virus may persist in the nose, and be recovered after 72 hours (Henle *et al*, 1946 *a*).

Volunteers can, however, be infected readily by inhalation of an atomized suspension, the virus "mist" can be administered in an inhaling apparatus or in a small experimental chamber It appears that the virus must, therefore, enter the tissues by the lower, as opposed to the upper, respiratory tract

In this type of work it is necessary to fix a criterion of what is to be regarded as a positive result It appears that a temperature rise to 100° F. (oral) and over may be accepted as evidence of infection

With influenza A strains a number of workers has secured infection by inhalation (Burnet and Foley, 1940, Burnet, 1942 *b*, 1943 *b*, Henle, Henle, and Stokes, 1943, *Francis et al*, 1944 *a*, 1945, *U S Nav Med Lab*, 1944 *b*, *Henle et al*, 1946 *a*)

For example, Henle *et al* (1946 *a*) found febrile responses in 58/65 persons after inhalation of 0 4 to 0 8 c c of finely atomized pure allantoic fluid Fluid diluted 10^{-1} or 10^{-2} was usually noninfective

The reaction to inhalation of virus A usually comes on within 24 hours, but may be delayed for 36 hours or more The fever seldom lasts for more than 48 hours. Common symptoms and signs are malaise, chills, headache, generalized aching and pains, flushing of the face, cough, and leukopenia Local coryzal symptoms may occur. Antibody rise occurs after experimental infection (see Ch LXIII) Subclinical infections may also occur, being shown by slight rises of temperature, leukopenia, or a rise in antibody

Virus can be recovered from throat washings after inhalation, for example by amniotic inoculation (see, e g, Burnet and Foley, 1940, Henle, Henle, and Stokes, 1943).

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

they found that the epithelium was resistant to influenza virus, to iontophoresis, and to zinc sulfate Later, resistance to virus remained, but the other agents acted as if in normal animals, although some degree of resistance to zinc sulfate remained for 5 weeks. They considered that this resistant state is nonspecific, but of temporary importance in immunity.

The upper part of the trachea appears normal, but there is a polymorph infiltration in the lower portion (Perrin and Oliphant)

In early passages, the lungs show reddish-purple patches, but with further passages become definitely consolidated According to Perrin and Oliphant, by the first day there is early bronchitis, pulmonary congestion, and infiltration of inflammatory cells. By the 2nd and 3rd days the bronchitis is purulent, with some degeneration of the epithelium. The alveolar exudate contains polymorphs and large mononuclears There is some perivascular cuffing. The pneumonic lesions are most pronounced in the bases of the lower lobes. The pneumonia progresses till the 6th day. After the 7th day there is evidence of proliferative change replacing the exudate, and this progresses so that by the 14th day the pneumonic areas are definitely smaller.

According to Sheftel (1940), after intranasal inoculation, encephalitis or meningoencephalitis may ensue.

Adsorption of influenza virus on cells of the respiratory tract The adsorption of influenza virus by red cells is probably due to specific receptors on the adsorbing cells (see Ch. LXI), and it is probable that the cells of the respiratory system possess similar receptors.

Hirst (1943 *a*) has investigated the problem by injecting allantoic fluid into the trachea of perfused and excised ferret lungs. Virus titers were studied by the chick cell agglutination (CCA) test and mouse infectivity. (1) Using PR8, almost all the virus was removed from the perfusing fluid in 5 minutes Elution was noted after 2 hours, and by 5 hours about 25 per cent of the amount adsorbed had been eluted In the case of the Lee strain, the release of virus was 75 per cent. complete in 5 hours. (2) After elution of virus, the cells did not appear capable of adsorb-

When examining ferrets 24 hours after inoculation, however, free agglutinin was readily detected, probably due to multiplication of virus after destruction of available receptors Hirst postulates that the destruction of a specific receptor substance may be a preliminary to parasitization of the susceptible cell by influenza virus This may involve an enzymatic reaction

Mouse

That mice could be infected was first shown by Andrewes, Laidlaw, and Smith

mice which, when passaged, produces extensive respiratory involvement (see also Gordon *et al*, 1938) Freeman (1940) has drawn attention to a spontaneous pneumonitis of mice which has some resemblance to influenzal infection It is distinguishable histologically by the absence of bronchiolar metaplasia and the presence of hepatitis

Mice are susceptible to infection with A, B, and swine strains of virus Friedewald and Pickels (1944), on the basis of centrifugation studies, concluded that approximately 10 infective particles are needed to infect mice with PR8, but 10,000

streptococcus (Group C) was transmissible in ferrets along with the virus, the double infection being more severe than that due to the virus alone. When streptococci were inoculated in animals previously exposed to virus, infection was caused

Course of the disease. The course of the experimental disease depends on the number of passages the virus has undergone, and whether the animal is etherized at the time of inoculation. As already mentioned, strains of virus recovered in the absence of severe epidemics are often difficult to pass to ferrets (see p. 583). When inoculated (without any etherization) with comparatively unpassaged strains, animals become ill after about 2 days The temperature rises to 105°–106° F. (normal 102° F.), and then falls; a characteristic feature is the appearance of a second "spike" of high temperature 2–3 days later, but this diphasic response may not be observed The animal is obviously ill, being quiet and weak It soon shows evidence of catarrh of the nose and eyes, and there is a purulent obstruction of the nasal passages with some discharge. Sneezing is a common feature and serves to disseminate the infection. The animal usually recovers in 7–10 days The addition of *Hemophilus influenzae suis* to the inoculum of virus does not render the attack any more severe (Smith, Andrewes, and Laidlaw, 1933).

When the animals are thus inoculated, the reaction appears to be localized to the upper part of the respiratory tract. Actually, however, virus may be present in [illegible] nd after 3 to 4 "passages-by-faith" [illegible] usually develops (Francis, 1935). The virus strain frequently requires a few passages before it begins to produce pneumonia regularly on the 4th to 5th day. Pneumonic animals suffer from a more severe illness than those presenting only catarrh, the breathing being labored and rapid. A similar pneumonic process develops when virus which has been frequently passaged is injected without ether (see Laidlaw, 1935).

Technique of passage. It is best to kill the ferret while still showing symptoms The turbinates are removed, ground with sterile abrasive, and a saline suspension is then centrifuged. A Gradocol membrane filtrate is prepared from the supernatant, for passage. If desired, lung suspensions can be similarly prepared

Pathology. The pathology has been studied by a number of authors (e g, by *Smith, Andrewes, and Laidlaw, 1933; Brightman, 1936;* McIntosh and Selbie, 1937 *a*, *b*, Perrin and Oliphant, 1940).

The nose is full of purulent exudate and the nasal mucosa is swollen and inflamed Microscopic examinations have been reported fully by Francis and Stuart-Harris (1938 *a*) and Perrin and Oliphant [illegible] Perrin and Oliphant, who worked with the PR8 strain, after 2 [illegible] congested and shows focal infiltration [illegible] lymphocytes. By the end of 48 hours, these changes are more marked and polymorphs are more numerous Over the turbinates, the *columnar ciliated epithelium* is replaced by a single layer of flat cells with small areas of denudation. By the 3rd or 4th day, both respiratory and olfactory mucosae are involved Changes vary from ferret to ferret, but usually not more than half the available area is involved

The process of repair begins by the 5th to 6th day, when there is definite *stratification of the polyhedral epithelial cells* of the respiratory mucosa By the 7th to 11th days, cellular infiltration is less, and most of the respiratory mucosa is covered by stratified transitional epithelium By the 19th day, most of the respiratory epithelium covering the turbinates and floor is stratified *columnar, while that* on the roof and walls is normal (Perrin and Oliphant)

If repeated inoculations are given, the mucosa shows a marked acceleration of repair (Francis and Stuart-Harris, 1938 *b*)

The epithelial changes that occur in the mucosa during recovery from infection were *found to* be associated with a functional alteration in the susceptibility to various substances (Stuart-Harris and Francis, 1938). On the 7th to 8th days,

they found that the epithelium was resistant to influenza virus, to iontophoresis, and to zinc sulfate Later, resistance to virus remained, but the other agents acted as if in normal animals, although some degree of resistance to zinc sulfate remained for 5 weeks They considered that this resistant state is nonspecific, but of temporary importance in immunity.

The upper part of the trachea appears normal, but there is a polymorph infiltration in the lower portion (Perrin and Oliphant)

In early passages, the lungs show reddish-purple patches, but with further passages become definitely consolidated. According to Perrin and Oliphant, by the first day there is early bronchitis, pulmonary congestion, and infiltration of inflammatory cells By the 2nd and 3rd days the bronchitis is purulent, with some degeneration of the epithelium The alveolar exudate contains polymorphs and large mononuclears. There is some perivascular cuffing. The pneumonic lesions are most pronounced in the bases of the lower lobes. The pneumonia progresses till the 6th day After the 7th day there is evidence of proliferative change replacing the exudate, and this progresses so that by the 14th day the pneumonic areas are definitely smaller.

According to Sheftel (1940), after intranasal inoculation, encephalitis or meningoencephalitis may ensue.

Adsorption of influenza virus on cells of the respiratory tract The adsorption of influenza virus by red cells is probably due to specific receptors on the adsorbing cells (see Ch LXI), and it is probable that the cells of the respiratory system possess similar receptors

2 hours, and by 5 hours about 25 per cent of the amount adsorbed had been eluted. In the case of the Lee strain, the release of virus was 75 per cent. complete in 5 hours. (2) After elution of virus, the cells did not appear capable of adsorbing any more (3) Heat- or formalin-inactivated virus was adsorbed but not eluted

Hirst (1943 *a*) also carried out experiments on the intranasal inoculation of living ferrets The animals were killed at varying periods after inoculation, and the lungs perfused and excised Buffer injected into the trachea was then tested for the CCA titer The virus was rapidly adsorbed, and after a period could not be eluted. When examining ferrets 24 hours after inoculation, however, free agglutinin was readily detected, probably due to multiplication of virus after destruction of available receptors Hirst postulates that the destruction of a specific receptor substance may be a preliminary to parasitization of the susceptible cell by influenza virus This may involve an enzymatic reaction

Mouse

That mice could be infected was first shown by Andrewes, Laidlaw, and Smith

mice which, when passaged, produces extensive respiratory involvement (see also Gordon *et al*, 1938) Freeman (1940) has drawn attention to a spontaneous pneumonitis of mice which has some resemblance to influenzal infection It is distinguishable histologically by the absence of bronchiolar metaplasia and the presence of hepatitis.

Mice are susceptible to infection with A, B, and swine strains of virus. Friedewald and Pickels (1944), on the basis of centrifugation studies, concluded that approximately 10 infective particles are needed to infect mice with PR8, but 10,000

with Lee (B). Himmelweit (1943) could not infect mice with the Paddington strain of virus B, and other strains have also proved noninfective.

Intranasal instillation. Mice are generally inoculated by dropping 0·05 c.c. of inoculum into the nares under light etherization. As a general rule mice can be infected only with ferret- or egg-passaged material, human washings usually failing to infect (Andrewes, Laidlaw, and Smith, 1935; and see p. 584). Certain strains, unusually virulent for the mouse, have, however, been isolated directly from washings; it is usually necessary to pass the lungs "blind" for a few times before lesions are found (Clampit and Gordon, 1937, Francis and Magill, 1937 *b*, Francis *et al*, 1937 *a*; and see p. 585). When lung suspensions are passed every 24–48 hours, there is a danger of bacterial pneumonia developing, probably due to the animal's nasal flora being washed into the lungs, this can be obviated by adding penicillin and streptomycin to the lung suspensions (McKee and Hale, 1947).

Following nasal instillation, virus rapidly invades the lung, and may also be isolated from blood, urine, bile, spleen, liver, and brain (Cerrutti, 1937; Smorodintseff and Ostrovskaya, 1937; Burnet and Lush, 1938 *b*; Bijl and van Genderen, 1939); it has also been detected in the mediastinal lymph glands (Burnet and Lush, 1938 *b*), and feces after 3 days (Sarracino and Soule, 1941 *b*). After intranasal inoculation, there is a large increase in virus in the lung within 20 hours, with a peak at 48 hours, little consolidation is present at this stage, but is well marked after 72 hours (Burnet and Lush, 1938 *b*). Taylor (1941 *c*) found that following the intranasal instillation of PR8, there is a rapid increase of virus in the lungs, and with large doses this becomes maximal within 24 hours; with smaller inocula the maximum concentration may not be reached till 48 hours after inoculation He made the interesting observation that even if a sublethal dose of virus is inoculated, the lungs may contain at least 78,000 MLD of virus, the mouse, however, survives

Following intranasal instillation of virus, death may ensue from pneumonia in from 3–7 days With weaker inocula, or less active viruses, the animal may survive and show varying degrees of pulmonary consolidation. Finally, if a sublethal dose is given, the animal remains apparently healthy.

An interesting phenomenon may be observed following intranasal inoculation. If a sublethal dose of virus is administered, and a second instillation of sterile water, Tyrode, saline, broth, or 10 per cent. normal serum is given after 4 days, there is a sharp rise in the virus content of the lung, and the animal dies in 3–8 days (Straub, 1940, Taylor, 1941 *c*) This effect can be produced if the 2nd injection is given up to 10 days after the first, but not if the second injection is of immune serum. Taylor suggests that the second inoculation "releases" virus already present in the cells of the respiratory mucosa to spread by the air passages and give rise to a true virus pneumonia These observations were confirmed by Harford, Smith, and Wood (1946).

The process of adaptation to mouse lung has been recently re-explored by Hirst, (1947 *a*) who suggests that it is commonly assumed that on initial transfer in a new host, a virus grows poorly, and only a few particles survive These survivors are held to be variants with greater capacity for multiplication in the new host Serial passage favors the emergence of further variants. The process of adaptation of influenza virus to mouse lung that he reports may, however, require some other interpretation of the adaptive process to be advanced

1 This experiment concerned an influenza A strain that was isolated by amniotic inoculation, and subsequently went through 4 allantoic passages The allantoic fluid of the 5th egg passage was inoculated nasally in mice After 3 days the mice were killed and lung suspensions passed to further mice, and so on in series Each mouse lung preparation and the original allantoic fluid were titrated in the egg and in mice Although the initial inoculum contained large numbers of egg infectious particles, it caused no deaths in mice However, the apparently normal lungs of these mice contained large amounts of virus as shown by egg-infectivity

tests, mouse lungs of the first passage contained as much virus as did those of subsequent passages By about the 6th passage in mice, lung suspensions were killing mice in high dilutions, and this level was maintained without further increase through many subsequent generations. In general, the titer of lung suspensions for eggs remained constant throughout the process of adaptation, and showed no marked tendency to increase.

2. In work with another strain of A virus, an attempt was made to correlate the hemagglutinin titer in the lungs with the titer in eggs Two trends were noted the agglutinin titer against chick cells did not increase with passage, but the titer against guinea-pig cells did, further, the guinea-pig chicken cell titer ratio was 1, but during passage was usually over 1, and increased to 128. It appeared as if the original D phase virus was becoming, on passage, more like Burnet's O form

Summarizing this interesting work, it has been shown that the enhancement of virulence for the mouse lung with mouse lung passage is not due to an increasing ability of the virus to grow in the lung. Rather, the egg-infecting type of virus seems to be gradually replaced by an entity having the same egg growth potentialities but t

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fatal, WS and PR8 strains were found to give rise to contact infection more readily than Melbourne or swine strains (Eaton, 1940 *b*). This type of infection may arise by inhalation of droplets, or by ingestion, as lung lesions may follow the introduction of virus by this route (Eaton)

Infection by inhalation Numerous workers have shown that mice can be infected by exposure to finely atomized virus suspensions (A or B) in a small closed b

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die. Animals are highly susceptible to infection by inhalation, and the course of the disease depends on the amount of virus inhaled Thus even 15 seconds exposure to as little as 0 2 to 0 3 c c of virus suspension diluted 10^{-2} dispersed in a chamber of 60 liters capacity was sufficient to produce pulmonary lesions in all animals; death regularly followed exposure for 2 minutes (Loosli, Robertson, and Puck, 1943) In these experiments, the infective droplets were calculated to measure 2–3 μ Knight (1946 *a*) infected mice in parallel by instillation and exposure to a spray. He found that the virus content of the lung, as measured by the agglutinating activity for chick cells, was usually about 50 per cent. higher in the animals infected by inhalation

Intracerebral injection The ordinary WS strain is not encephalitogenic (Daddi and Panà, 1938) Stuart-Harris (1939) has produced a variant of this strain by egg passage which gives rise to encephalitis in mice, and can be passed by the intra cerebral route Francis and Moore (1940) confirmed these observations, but found it unnecessary to pass the strain through eggs, they used a tissue culture as the source of virus They found that the Melbourne strain produced similar changes, but not swine or several other strains

Neurological signs ascribed to toxic properties in the influenza virus have been observed by certain workers (see below).

Injection by other routes Mice can be infected by direct injection into the lungs, through the chest wall (Andrewes, Laidlaw, and Smith, 1934).

Following intraperitoneal or intravenous inoculation of large doses, virus can be recovered from the lungs, after subcutaneous inoculation, virus can be detected in the regional lymph glands (Rickard and Francis, 1938, Henle and Henle, 1946 *b*).

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pear dilated. There is edema of the peribronchial tissue with slight monocytic infiltration The first result of the dilatation of the respiratory bronchioles is to give emphysema. Later, the alveoli become collapsed, and edema and congestion give rise to areas of consolidation. Collapse is a complication only, and not a specific change; it may be absent in adult mice or those infected with a weak virus.

Straub describes the reparative lesions beginning about the 5th day, when the larger bronchi return to normal, in the bronchioles the epithelium proliferates and becomes almost stratified (see also Laidlaw *et al*, 1935). This overgrowth fills the terminal air spaces, so that eventually the involved area becomes functionless.

Straub's findings with virus A have been confirmed by de Balogh (1939) and others (Nelson and Oliphant, 1939, Hoyle and Orr, 1945; Harford, Smith, and Wood, 1946). de Balogh reports necrosis and desquamation of alveolar epithelium, and evidence of motor dysfunction of the muscular tissue of the bronchioles, pulmonary arteries and arterioles, due to lesions in the ganglion cells of the bronchial wall.

Nelson and Oliphant infected mice by the intranasal instillation of PR8 virus and examined the lungs at frequent intervals. Signs of bronchial epithelial damage and bronchitis were noted even after 24 hours. Epithelial damage was more marked after 48 hours, when the early polymorph exudate in the bronchi was necrotic. There was perivascular edema and peribronchial and perivascular infiltration with small mononuclears After 3–4 days they found a diffuse edema, and a septal and alveolar infiltration with polymorphs and mononuclears Some bronchial epithelium was denuded Animals surviving for 6 days lived indefinitely and showed squamous metaplasia in the alveoli

Hoyle and Orr studied the changes from day to day after intranasal instillation of WS They found slight desquamation and inflammation of the bronchial epithelium after 24 hours. After 48 hours there was well-marked epithelial degeneration, with plugs of mucopus in the lumina, focal bronchiolar dilatation, and peribronchitis. After 72 hours there was focal denudation of the bronchial wall After 4–6 days, regeneration of the bronchial epithelium was seen, with stratification They concluded that the alveolar changes resembled those seen in bacterial infections.

The histopathology of infection with virus B has been reported by Oliphant and Perrin (1942), who found that the changes are essentially as seen with virus A. There are degenerative changes in the bronchi, followed by reparative proliferation In the late stages there is plugging of the alveoli by epithelial cells of squamous type

The histological features after the intranasal inoculation of swine virus are fully described by Dubin (1945), who likens them to those due to human strains He found necrotic changes in the bronchial and alveolar lining, often with formation of a hyaline membrane The bronchial changes give rise to collapse. Proliferative repair in the bronchi and bronchioles is very marked, and cells invade and fill the alveoli. About the 14th day many alveolar plugs degenerate and reopen the alveoli. The epithelium of the bronchi and bronchioles is restored to normal in the 3rd week There is, in addition to the bronchitis, a pneumonia characterized by hyaline necrosis of alveolar cells, congestion and focal hemorrhage, edema, and interstitial infiltration with mononuclears

Sheftel (1940) has described the presence of encephalitis and meningo-encephalitis after intranasal infection.

Influenza virus appears to have a somewhat inhibitory effect on the development of primary lung tumors occurring in mice after exposure to dust (Campbell, 1940)

An interference phenomenon The cerebral inoculation of large doses of WS virus mixed with western equine virus interferes with the proliferation of the western virus PR8 and Lee also interfere (Vilches and Hirst, 1947).

Technique of autopsy on mice. When it is necessary to examine the lungs of large numbers of mice, as in titration experiments, and particularly when they are to be removed with aseptic preparations, the technique of Himmelweit (1944) can be recommended: the mouse is pinned to a board on its back. The skin over the lower abdomen is picked up with forceps and "nicked" with scissors. The skin can then be torn upwards and laterally by traction with the forceps, exposing the abdomen, thorax, and root of the neck. This flap is then pinned down. These nonsterile instruments can be used for a whole series of mice. The exposed thoracic wall can be regarded as sterile, and is then opened with a second (sterilized) pair of instruments, and the lungs removed.

When examining lungs for pneumonic lesions, it is important to remove the organs from the thorax, as the heart covers most of the lung surface *in situ*. Alternatively the thorax can be opened from the back.

Influence of physical and chemical agents on infection. Mice kept at 95° F., after intranasal instillation of PR8, showed lesser degrees of pulmonary consolidation than those kept in a cold room (Sulkin, 1945).

Alcohol, heat, cold, and fatigue did not render animals more susceptible to infection (Sarracino and Soule, 1941 *a*).

The intraperitoneal administration of atropine sulfate shortly before the nasal instillation of PR8 decreased the incidence and extent of infection, perhaps because of the consequent reduction in mucous secretion (Wheeler and Nungester, 1944).

Combined virus and bacterial infections. A number of authors has described the effects of subjecting mice to double infections with bacteria and organisms. Thus, Herzberg and Gross (1940) were able to pass a Type 3 pneumococcus and influenza virus serially in mice without loss of virulence of either component.

Experiments have been conducted by exposing mice to atomized bacteria after infection with virus A. For example, Wells and Henle (1941) administered Type 1 pneumococcus suspension 1–5 days after a fatal dose of virus, and found that death occurred more quickly with the double infection. When sublethal doses of virus were employed, there was an increased mortality rate and a greater incidence of severe lesions in those animals with the double infection. Similar observations have been made by Francis and de Torregrosa (1945) using *H. influenzae*, staphylococcus, and hemolytic streptococci; they found that these organisms invaded the lungs of mice only if superimposed on a preexistent virus infection. Harford, Smith, and Wood (1946) found that when mice showing reparative changes in their bronchial epithelium due to virus infection were exposed to atomized bacteria, there occurred polymorph infiltration and alveolar edema in addition to the virus changes.

Ballowitz (1944 *b*) infected mice with paratyphoid bacilli by the mouth, and then instilled virus nasally. He found that under these conditions general dissemination of the organisms was facilitated (see also 1944 *a*).

PR8 was found to enhance the development of lesions in the lungs due to infection with tubercle bacilli (Volkert *et al.*, 1947).

Pathology. The infection in the mouse is mainly localized in the lungs, whereas in the ferret the changes are chiefly in the upper respiratory tract. Naked-eye the lungs show varying degrees and extent of consolidation. Part of one lung only may be involved, or in severe infections both organs may be "solid." The affected areas are firm and purple, and depressed below the surface of the remaining emphysematous portions.

Although the histological features were studied by earlier workers, Straub (1937, 1939, 1940) using the WS strain has furnished one of the most complete accounts. He finds that influenza virus affects specifically the epithelium of the respiratory tract from the bronchioles to the bifurcation of the trachea. The primary change is necrobiosis and fibrinoid necrosis of the bronchiolar epithelium. The terminal and respiratory bronchioles are denuded of their epithelium and ap-

thickening due to proliferation of endothelial cells O phase virus is more virulent than D phase (see Ch LVI)

Woolpert *et al* (1938) have shown that the fetal guinea-pig can be infected cerebrally with the PR8 strain of virus The virus could be recovered from the fetal lung, liver, blood, and brain as well as from the placenta (see also Dettwiler, Hudson, and Woolpert, 1940).

Hedgehog. Stuart-Harris (1936) found that a mild, but contagious respiratory infection could be produced in hedgehogs, and he was able to pass the infection in series The animal should be inoculated intranasally.

Rabbit. Intranasal inoculation gives rise to an inapparent infection (Hyde, 1942), McIntosh and Selbie (1937 *a*, *b*) recorded the presence of lung lesions resembling those of guinea-pigs Cerruti and di Aichelburg (1937) found that the virus survives subclinically in the brain after intracranial injection, while Daddi and Panà (1937) found evidence of inflammatory reaction.

Monkey. Intranasal inoculation may produce mild signs of infection (Vieuchange, 1939), direct intratracheal injection in cynomolgus monkeys may produce a fatal virus pneumonia characterized by bronchiolitis and interstitial changes (Burnet, 1941 *b*) Extensive experiments with *M. mulatta* have been carried out by a group of investigators (Merino *et al*, 1941, Woolpert *et al*, 1941, Saslaw *et al*, 1946, Wilson *et al*, 1947) with the following conclusions

1. Nasal inoculation in normal animals may produce a subclinical infection characterized by leukopenia A much more severe and usually fatal infection with lung changes occurs in nutritionally deficient animals, or animals exposed to cold

2 Intratracheal injection may produce signs of infection and leukopenia, severe in nutritionally deficient animals.

3. Simultaneous nasal inoculation of normal animals with virus and streptococci failed to produce evident disease Most of the animals showed a virus-induced leukopenia preceded by a leukocytosis due to the streptococci

4 Inoculation of streptococci 4–17 days after the virus produced disease in some animals, and a few developed fatal streptococcal septicemia.

5 A number of monkeys inoculated with both agents developed glomerular nephritis

6 Reinoculation with streptococci caused a rise in opsonins, precipitins, and antistreptolysin titers Administration of virus prevented this effect

7. It was concluded that virus infection predisposed to secondary bacterial invasion, and depressed the cellular and humoral defense mechanisms

[illegible] embryo chick it is surprising that [illegible] before show no pathological cha[illegible] hatching is the factor determining resistance (Enders and Pearson, 1941)

Chinese mink Tang (1938) found that *Mustela sibirica* (Milne-Edwards) is susceptible to the WS strain, after intranasal inoculation a febrile response develops. With passage, extensive pneumonic consolidation occurs

Miscellaneous The following have all been reported susceptible to influenza virus

1 David's squirrel—*Sciurotamias davidianus* (Milne-Edwards) (Tang, 1938).

2 Corean squirrel—*Eutamias asiaticus orientalis*, and

3 Manchurian squirrel—*Citellus mongolicus umbratus* (Thomas) (Tsurumi, Ogisawara, and Takagi, 1939)

4 Kangaroo rat—*Dipodomys* (Eaton, Martin, and Talbot, 1941)

5 *Galictis furax furonax* (Taylor, Parodi, and Chialvo, 1942–3)

6 A group of Tunisian rodents—*Gerbillus hirtipes, Dipodillus campestris, D. dodsoni*—are all said to be as susceptible as the mouse, *Meriones shawi* and *Jaculus jaculus* are less susceptible (Durand, 1945).

Pig

Human influenza strains, e.g., WS and PR8, can be transmitted to the pig by nasal instillation under light etherization (Elkeles, 1934; Shope and Francis, 1936, Tulloch, 1939, Mote and Fothergill, 1940). Shope and Francis concluded that the intranasal inoculation of human virus causes a mild illness with scanty lesions in the lungs, this illness is similar to that produced by the swine virus When *H. suis* and human virus are administered together, an illness resembling a mild attack of swine influenza develops, pneumonia is found, but is less severe than that of true swine influenza.

Certain evidence has been obtained which suggests that pigs may become infected naturally with human virus. Thus, Shope (1938) investigated the serum antibodies of pigs in American farms on which workers had suffered from epidemic influenza in 1936–7. Two classes of pig were examined, those that had lived through the 1936–7 epidemic, and those born subsequently. In the older pigs he found virus neutralizing antibodies to the human virus, while the young pigs' sera contained no antibodies to this virus. As these older animals were, and had always been, in perfect health, he suggested that they had acquired a subclinical infection with a human virus (see similar observations of Shaw *et al.*, 1939).

Hamster

Various workers have reported on the susceptibility of Syrian hamsters to influenza virus, and their value in diagnostic work (see p 584).

The European hamster (*Cricetus cricetus*) can be infected with ferret-passed material by intranasal inoculation, but not with human material (Taylor and Dreguss, 1940 *a*).

The Chinese hamster was found to be susceptible to strains of virus A in early mouse passages, the incubation period is 3–6 days, and death occurs after 4–10 ... onsolidation (Yen, 1940).

... that virus A suspended in mucin injected ... the lungs, similar to those found in mice.

Other Animals

Rat Various workers have attempted to infect the rat by intranasal inoculation of human washings or passage virus (Agapov, 1936, Stuart-Harris, 1937 *b*, Valls Conforto, 1938, Hyde, 1942) After inoculation of washings, the animals do not show marked symptoms, but develop a subclinical illness, virus can be recovered from the nose and lung (Stuart-Harris) A fatal infection may follow the inoculation of ferret-passed material (Valls Conforto).

Harford *et al* (1946) have produced a mild infection in rats by direct intrabronchial inoculation with PR8 virus The animals were etherized and a cannula passed through the larynx under direct vision, and thence into the left main bronchus. A catheter was passed through this cannula into the lower part of the left lobe, so that the inoculum was consistently placed close to the lower pole Lesions occurred near the point of inoculation The area involved was dark red and firm Microscopically, there was edema and cellular infiltration of the peribronchial and perivascular tissue, and infiltration of cells into the walls and lumina of the alveoli Twenty-four hours after inoculation, most of the infiltrating cells were polymorphs, but after 4 days large mononuclears predominated There was no necrosis of epithelial cells—an unusual finding.

Guinea-pig Like the ... infection (Stuart-Harris, 1937 *b*, Bijl and ... may be recovered from the nose only, bu ... patches of collapse and hemorrhage in the lungs, the alveoli contained serous exudate, and there was

¼, +, ½, ++; ¾ consolidated, +++. Fifty per cent. end points are then calculated (Reed and Muench), 3 different criteria being employed, (*a*) death due to influenza within 10 days, (*b*) development of pulmonary consolidation within 10 days, (*c*) a weighted composite taking account of death and the extent of consolidation. It was found that the chances are 19/20 that differences of 0 99, 0 77 and 0 73 logarithmic units respectively for the lesion, the death, and the weighted end-points are significant.

Knight (1944 *b*) compared the accuracy of titration by inoculation of mice and chick embryos, in parallel. He found that accurate results can be obtained when 10 embryos or mice are used per dilution, but sufficient accuracy is achieved with 5. He found that the embryo is sensitive to considerably smaller amounts of virus than mice. (Titrations in eggs are fully described in Ch. LIX, and by red cell agglutination in Ch. LXI).

LABORATORY CONTAMINATION WITH INFLUENZA VIRUS

Andrewes *et al* (1944) described a number of incidents where accidental contamination with laboratory strains of influenza virus occurred during transmission experiments The contaminating virus may have gained entrance due to inadequate sterilization of instruments or glassware, or from dust.

7. Cane rats–*Zygodontomys microtinus stellae*, and
8. Bush rats–*Proechimys cayennensis o'connelli* (Allen) (Weir, 1944).

TOXIC EFFECTS OF VIRUS IN ANIMALS

It has been suggested that some strains of influenza virus may, under certain conditions, produce effects by virtue of a toxin rather than by reason of its invasive powers.

Thus neurological signs such as tonic and clonic convulsions, produced in mice by the intracerebral inoculation of concentrated preparations, have been ascribed to a toxin. These signs come on after 12–48 hours, and the animals die after 24–72 hours. Histologically, there is destruction of the ependyma of the ventricles. There *is no evidence of virus multiplication and passage cannot be performed (Henle* and Henle, 1944 *c*, 1946 *a*, Hale and McKee, 1945 *b*). The toxic effect can be abolished by ultraviolet rays, heat, or formalin, and neutralized by specific antiserum (Henle and Henle, 1944 *c*, 1946 *a*). Inoculation of virus A and B in the rabbit's eye produced corneal opacity, there was no multiplication of virus, and the effect was neutralized by type-specific serum (Evans and Rickard, 1945).

Toxic changes can also be shown in mice injected intravenously or intraperi- [illegible] in 18–46 hours with necrosis of the liver and spleen, [illegible] emorrhage in the gastro-intestinal tract, this effect [illegible] (Henle and Henle, 1945 *c*, 1946 *b*). Some die after 6–8 days with pulmonary consolidation; this effect is more pronounced with dilute preparations of virus; in some mice jaundice may develop (1946 *b*).

Henle and Henle (1946 *a*, *b*) have found that the toxic factor cannot be separated from the virus particle, and has the following properties· It is not dialyzable through cellophane, it is sedimented with the 600 S and not the 30 S component, it can be adsorbed to, and later eluted from, chick red cells, it can be precipitated *by protamine, it resists storage at 4° C. for 2–3 months; it withstands freezing* and thawing. Mice were immunized with allantoic fluid inactivated by UVL and rendered resistant to a challenge inoculation of the homologous toxic factor (*1946 b*).

In eggs, these workers found (*1946 b*) that the infective titer of virus in allantoic fluid often reached its peak within 24 hours, whereas maximum toxicity was not reached before the 2nd day. The application of heat, formalin, or irradiation with UVL caused more alteration in infectivity than in toxicity (see also 1947). There seems little doubt, however, that influenza virus has toxic properties, and that the virus particle itself carries the toxic property Toxicity and infectivity are possibly based on different constituents of the particle

TITRATION OF INFECTIVITY OF INFLUENZA VIRUS

It is often desired to assay the potency of virus preparations *in vivo*, and it is usual to carry out such infectivity tests by intranasal inoculation in mice A simple method is to make serial tenfold dilutions of virus, e g, of allantoic fluid in 10 per cent. serum-saline, and inoculate 0 05 c c of each dilution intranasally in 6 etherized mice. The 50 per cent end point is then calculated by the method of Reed and Muench (1938), taking as the end point of the titration death, or any desired degree of pulmonary consolidation, arbitrarily scored as from ± to ++++, occurring within 10 days of inoculation Horsfall (1939) has elaborated this type of test somewhat by calculating both the 50 per cent mortality end point and the 50 per cent. maximum score end point In this method account is taken of the extent of lung involvement as well as of death (see also Eaton, 1940 *a*).

A still more elaborate method of titration is that of Lauffer and Miller (1944 *a*) Serial tenfold dilutions are made in 0 1M phosphate buffer, pH7, and 6 successive dilutions, covering the expected end point, are inoculated intranasally in mice in 0 05 c.c. amounts. After 10 days, survivors are autopsied The degree of involvement of the lungs is scored as follows no consolidation, –, slight consolidation ±,

seen in purified virus preparations, these particles occur abundantly in control preparations from normal allantoic fluid, and would not appear to be related to the virus itself (Taylor *et al*, 1943 *b*).

Filtration

The virus passes the following simple filters: Seitz, Berkefeld V and N (Francis, 1934), Chamberland L_2 and L_5 (Brightman, 1936, Dujarric de la Rivière and Chevé, 1937 *a, b*)

Gradocol membranes are more widely used in preparing infective filtrates Using these membranes Elford, Andrewes, and Tang (1936) estimated the size of virus A and swine influenza to be between 80 and 120 mμ Friedewald and Pickels (1944) made confirmatory observations using the PR8 and Lee (B) strains

Virus is adsorbed by various earths and clays used in filters, the adsorbed virus can be recovered by treatment with a protein solution such as broth, serum, or isinglass (Hare and Curl, 1947)

Centrifugation

The behavior of influenza virus preparations when exposed to centrifugal force has been extensively studied An air turbine instrument of the Bauer and Pickels type has been much used, and the Sharples centrifuge can also be employed (see, e g, Stanley, 1944 *a*). When centrifuged in electrolytic solutions the rate of sedimentation is often irregular, presumably due to convection or mechanical disturbances Friedewald and Pickels (1943, 1944) have introduced a synthetic density gradient technique with sucrose solutions, sedimentation behavior is more regular in this medium (see also Stanley, 1944 *b*)

The fractions that can be isolated from virus preparations, especially allantoic fluid are 3—the elementary body, the soluble substance, and another complement fixing component known as < 30 S The elementary body, which possesses all the attributes of infectivity, is often known as the 600 S component, although the sedimentation constant varies somewhat with the type and presumably the technique employed The soluble substance has a sedimentation constant of 30 S The properties of the 3 components have been fully described above (see Ch. LVI)

The size of influenza virus can be calculated from sedimentation data Elford and Andrewes (1936) estimated the size of virus A to be between 87 mμ and 99 mμ. The results obtained by various later workers are in agreement with this estimate As regards the elementary bodies of virus A, sedimentation constants of from 600–724 S have been obtained, giving estimates of the particle diameter from 70–100 mμ (Taylor *et al.*, 1943 *b*, Beard *et al*, 1944, Friedewald and Pickels, 1944, Lauffer and Miller, 1944 *b*, Stanley, 1944 *a, b*, Lauffer and Stanley, 1944, Sharp *et al*, 1944 *b, c*, Knight, 1945, 1946 *a*)

The sedimentation constant of virus B seems to be higher (832 S), giving a size of 85–100 mμ (Sharp *et al*, 1943, 1944 *b, c*, Beard *et al*, 1944, Friedewald and Pickels, 1944)

Swine influenza viru [illegible]
found to be from 622– [illegible]
1944, Taylor *et al*, 194 [illegible]

The calculation of [illegible] re-
quires an estimate of various other biophysical properties. Lauffer and Stanley (1944), for example, give the following figures (PR8 strain). specific volume, 0 79, density in sucrose, 1 18, without sucrose 1 1, intrinsic viscosities, 11 3–16 5, water content, 60 per cent by weight More recently Sharp *et al* (1944 *a*, 1945), have introduced an improved technique in which the virus is centrifuged in bovine albumin This appears to give more regular results than centrifugation in sucrose The densities observed by this method were lower than those obtained by the sucrose technique They postulate that when virus is suspended in sucrose, water

CHAPTER LVIII

PHYSICAL AND CHEMICAL PROPERTIES OF INFLUENZA VIRUS

MORPHOLOGY AND BIOPHYSICAL PROPERTIES

Elementary Bodies

IT HAS *already been explained that influenza virus consists of elementary bodies* and a soluble substance. The elementary bodies are probably composed of several antigens as well as containing some SS, and material derived from the host. It has been suggested by Sharp *et al.* (1945)[1] that the elementary bodies are surrounded by a semipermeable membrane, as is found in bacterial cells. The elementary bodies of influenza measure about 100 mμ as has been determined by various methods (to be described). When stained, therefore, these bodies may be just visible with ordinary microscopic equipment. Gordon (1922) demonstrated small granules, 0.2 μ in diameter, in the secretions in the early stages of influenza, and more recently Japanese workers have studied similar bodies. They have found elementary-body-like granules in smears from the lung lesions of mice, and have concentrated them by centrifugation (Tsurumi and Ogasawara, 1940, Tsurumi, Ogasawara, and Fujii, 1941, Tsurumi *et al.*, 1941 *a*). These granules grow in tissue culture and form intracytoplasmic inclusions, they can be agglutinated by antiserum (Tsurumi *et al*, 1941 *b*)

Characteristic inclusions have not been described except by the Japanese workers.

Electron Microscopy

All 3 influenza viruses have been studied by electron microscopy, using as a rule material purified by centrifugation from infected allantoic fluid

Virus A elementary bodies appear to be rounded, ovoid, with a definite tendency to kidney or bean shape, they often occur in aggregates, and may show areas of greater density. Slightly varying estimates of the diameter of these particles have been made 78–80 mμ (Taylor *et al*, 1943 *b*, Beard *et al.*, 1944), and 100 mμ (Lauffer and Stanley, 1944, Knight, 1944 *c*, 1945, 1946 *a*).

An earlier estimate of a much smaller size has been explained by the presence of larger particles as a contaminant (Chambers and Henle, 1943, Chambers *et al*, 1943, Henle and Wiener, 1944).

Virus B elementary bodies appear rounded or ovoid, and have been estimated to measure 97–98 mμ (Sharp *et al*, 1943, 1944 *b*, *c*, Beard *et al*, 1944)

The swine influenza virus has also been studied, and appears to be rounded or *ovoid, measuring 77–78 mμ in average diameter (Beard et al.*, 1944, Sharp *et al*, 1944 *c*, Taylor *et al*, 1944 *b*).

The new gold-shadowing technique gives very striking pictures, and small clusters and chains can be seen (Williams and Wyckoff, 1945, Stanley, 1946) Mosley and Wyckoff (1946) describe the presence of elongated forms, as well as spheres. The elongated forms occur more frequently, and are longer, in preparations made from the Weiss strain. These rod-like forms may be in clumps together with spheres, or may lie separately, occasionally they lie in tangled masses. Most of the long forms show swellings, terminal, or bud-like. Several spheres may be attached to one rod. Normal allantoic fluid does not present these appearances

In addition to the elementary bodies, smaller particles, about 15 mμ, have been

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

The iso-electric point of Knight's normal allantoic protein is 2.3, and of normal mouse lung particles around 4.2 (Knight, 1946 *a*).

PURIFICATION AND CONCENTRATION

Influenza virus has been isolated in a high state of purity from mouse lungs and allantoic fluid. The following are the chief methods.

Purification by Freezing and Thawing

When allantoic fluid is frozen, and then carefully thawed, a fluffy white precipitate forms which goes into solution at 37° C From 75–95 per cent. of the virus is carried down on the precipitate, and can be eluted therefrom (Hare, McClelland, and Morgan, 1942, Hirst, Rickard, and Whitman, 1942)

Maximum yields of virus are obtained using a phosphate or citrate buffer at pH5 5 Some nonvirus material also goes into solution, but this is reduced to a minimum if the allantoic fluid is adjusted to pH8.8 (with NaOH) before freezing.

Purification by Adsorption to, and Elution from, Red Cells

Francis and Salk's (1942) method is widely used. In their original method chorioallantoic vessels are deliberately torn during the harvesting of the allantoic fluid. The red cells

is eliminated

A definite correlation exists between the cell concentration and the amount of virus adsorbed Thus Hare, Curl, and McClelland (1946) found that the amount adsorbed rapidly decreases when the cell concentration is below 1 per cent. Using a 2 per cent suspension, virtually all A and B virus is adsorbed. Within limits, variation in the volume of the eluting fluid does not affect the yield of virus, although of course altering the titer Under laboratory conditions, the efficiency of recovery of virus by the combined

13–80 per cent.

t 20,000 g for

virus can be

cent. of the

original infectivity (Sharp *et al.*, 1943, 1944 *b*; Beard *et al*, 1944).

Knight (1945, 1946 *a*), obtained a purified preparation of PR8 virus from mouse lungs by a technique involving storage at −70° C, thawing of the suspension, addition of chick red cells, elution in phosphate buffer at 37° C for 90 minutes, and final centrifugation of the eluate These preparations gave 50 per cent. end-points at 10^{-12} to $10^{-11.8}$ and 10^{-12} to $10^{-14.2}$ gm. of nitrogen in mice and chicks respectively The highest chick cell agglutinating activity of mouse lung preparations was 30,000 units per mgm of nitrogen Material obtained from allantoic fluid by similar means gave 50 per cent. end points in chicks of $10^{-14.3}$–$10^{-14.4}$ nitrogen, and in mice of $10^{-9.9}$–10^{-10} gm The chick cell agglutinating activity of these preparations was 40,000 units per mg of nitrogen He found that mouse lung virus has essentially the same properties as allantoic fluid virus, and is precipitated strongly by antiserum to PR8 grown in the allantoic cavity

Purification by Chemical Precipitation

1 Salk's method Virus can be adsorbed, e g, from a mouse lung suspension, by a

water (Salk, 1941, Schaeffer, 1942)

The calcium phosphate precipitate must be formed in the presence of the

To obtain the best yields by this method, the virus-containing material should

purified by differential centrifugation (Stanley, 1945 *a*)

2 Potassium urate adsorbs virus from allantoic fluid without loss

manovich, Parodi, and Chialvo, 1942 3)

3 Virus can be precipitated by alum, and the precipitate redisso'

is removed. It appears as if virus particles are surrounded by a semipermeable membrane permeable to sucrose but not bovine albumin. This membrane is similar in certain properties to the cell wall of bacteria. Particles placed in sucrose would first lose water and thus increase in density. At the same time sucrose would be in sufficient concentration to reverse the flow of water, this would result in a subsequent decrease in density. These authors have obtained the following figures in their studies of PR8, Lee, and swine viruses

TABLE 24

	PR8	*Lee*	*Swine*
Sedimentation constant	742	840	727
Density in aqueous suspension	1.104	1.104	1.100
Diameter calculated by cfg (in mμ)	*116*	*124*	*117*
Partial specific volume	0.822	0.863	0.850
Water content, per cent by volume	52.0	34.5	43.3

These figures for size are larger than obtained by workers mentioned above, and this can be explained by the different technique. The smaller estimates alluded to above were obtained with partly dehydrated particles, whereas in bovine albumin the particles can be regarded as fully hydrated.

Light Absorption

Virus solutions exhibit visible opalescence due to light scattered by virus particles. The molecular weight (M) of uniform spheres such as virus particles = $1.69 \times 10^{24} \dfrac{D\lambda^4}{c\left(\dfrac{\partial n^2}{\partial C}\right)^2}$ where D is the optical density, λ the wave length of light, c the concentration of particles in grams per c.c. of solution, and n *the index of refraction* (Oster, 1946). Using virus obtained from allantoic fluid, the MW was found to be 322 million, using a figure for density of 1.1, the diameter could be calculated at 97 mμ.

Analytical Diffusion

Bourdillon (1941), working with mouse lung preparations infected with virus A, found 99 per cent of the infectivity to reside in particles 200 μμ in diameter, and 1 per cent in particles measuring 6 mμ.

Electrochemical Behavior

Electrophoresis Bourdillon (1940) investigated the electrophoretic mobility of virus A and found it to be -5.5×10^{-5} c.m^2 volt^{-1} sec^{-1} at pH7–9, ionic strength 0.1, at pH10, ionic strength 0.04, it was about -8×10^{-5}.

Miller, Lauffer, and Stanley (1944) investigated the electrophoresis of crude preparations of PR8 obtained by high speed centrifugation. They identified 2 components One component, present to the extent of 80–90 per cent, represented the active virus, and could be separated from the other component by repeated fractional centrifugation, when it appeared homogeneous in the analytical centrifuge, and the Tiselius apparatus, the iso-electric point was at pH5.3 The second component, present to the extent of 10–20 per cent, was identified as an impurity similar to, or identical with, the substance isolated by Knight from normal uninfected as well as infected embryos

Iso-electric point Knight (1945, 1946 *a*) investigated purified PR8 virus obtained from mouse lung, and found that it had two electrochemical natures, one tending towards an iso-electric point at pH5.4, and the other at 7, pH5.4 is the iso-electric point of purified virus obtained from allantoic fluid As just mentioned, Miller, Lauffer, and Stanley (1944) obtained a figure of

polysaccharide composed of mannose, galactose, and glucosamine units. It was tentatively suggested that the latter component was derived from the host alone.

Knight (1947 *b*) attempted to discover a chemical basis for the similarities and differences between the PR8 and the Lee strain. Hydrolysates of the purified viruses were obtained, and assays made for amino acid content. No significant differences were observed with respect to the values for alanine, aspartic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine. Significant differences were found in the values for arginine, glutamic acid, lysine, tryptophane, and tyrosine. These differences may account for the lack of immunological relationship between the strains, for their different pH stability ranges, their different red cell agglutinating capacities, and the widely divergent heat stability of their hemagglutinins.

A considerable proportion of the volume of influenza virus is occupied by water, which is about 52 per cent. in the case of PR8, 35 per cent. for Lee (B), and 43 per cent. for swine virus (Sharp *et al.*, 1945).

virus can first be concentrated by adsorption on chick cells (Bodily, Corey, and Eaton, 1943)

4. Virus can be precipitated by protamine (Chambers and Henle, 1941).
5 Calcium sulfate also has been used (Tovarnizky and Chalkina, 1944).
6. Virus can be purified with ethyl or preferably methyl alcohol as a preliminary to high speed centrifugation (Cox *et al*, 1946, 1947).

Purification by Electrophoresis

Purification can be effected by electrophoresis (Davoli and Jucker, 1939, Miller, Lauffer, and Stanley, 1944; Tovarnitski and Shishkina).

Purification by Centrifugation

It appears that the most efficient method of purifying virus is by differential centrifugation in a vacuum type of centrifuge, or a Sharples machine. Thus Stanley (1944 *a*) studied the following methods of purification of PR8 grown in the allantoic cavity.

1. Differential centrifugation in a vacuum or Sharples type of centrifuge.
2 Adsorption to, and elution from, adult chick red cells
3 Elution of the precipitate formed on freezing and thawing of the allantoic fluid
4 By adsorption to, and elution from, the chick red cells of the egg yielding the allantoic fluid.
5 Combinations of (1) with (2), (3), and (4).

He found that No. (1) was the most efficient, and yielded much less nonspecific protein He obtained a fairly homogeneous compound with a sedimentation constant of 600 S Such preparations possessed about 22,000 chick cell agglutinating units per mg of protein nitrogen, and the 50 per cent infectivity end-point in chick embryos was 10^{-11} gm

McLean *et al* (1945 *d*) concentrated swine virus by centrifugation in the Sharples instrument. The average infectious unit of virus when inoculated in 0 05 c c volumes in chick embryos was $10^{[illegible]}$ gm of virus, $10^{[illegible]}$ gm gave the ++ end-point in CCA tests The degree of concentration was 130 times, and the yield of virus of 2 2 mgm per 100 c c. of allantoic fluid

CHEMICAL COMPOSITION

Various workers have analyzed the chemical composition of all 3 strains of influenza virus isolated from mouse lung or allantoic fluid. Such preparations contain nitrogen, phosphorus, carbohydrate, fat-solvent extractive (neutral fat, phospholipoid, and cholesterol), nucleic acid (desoxypentose type) (Beard *et al*, 1944, Cohen, 1944, Sharp *et al*, 1944 *b*, Taylor, 1944, Taylor *et al*, 1944 *b*, Knight, 1946 *a*, Miller, 1947)

Slight differences have been detected in the composition of the 3 main strains The main difficulty in this work is the closely similar chemical composition of normal allantoic protein, and normal mouse lung particles Thus Knight (1946 *a*) gives the following details concerning the chemical composition of virus purified from 2 sources, compared with the composition of normal material.

TABLE 25

	PR8 Purified from Mouse Lung	*PR8 Purified from Allantoic Fluid*	*Normal Allantoic Protein*	*Normal Mouse Lung Particles*
Nitrogen per cent	10 1	10 2	9	6 9
Phosphorus per cent	1 06	0 92	0 81	1 4
Carbohydrate as glucose	6 2	6 5	7 7	3 5
Alcohol-ether extractable substance	38 (approx)	26 8	22 4	
Ribonucleic acid	+	+	+	+
Desoxyribonucleic acid	+	+	+	doubtful

In later observations Knight (1947 *a*) obtained both ribonucleic and desoxyribonucleic acids from purified PR8 particles These particles contained carbohydrate additional to that of nucleic acids Lee and PR8 particles seemed to contain a

embryos showed numerous hemorrhages in the muscles, skin, and brain. The WS strain also has been fully adapted to the chorio-allantois (Burnet and Lush, 1938 *b*). The strain underwent a sharp increase in titer between the 41st and 50th passages The foci were then particularly liable to be surrounded by "secondaries." When titrated by pock-counting, there is a simple linear relationship between the concentration of virus (WS egg) and the number of foci produced (Burnet and Faris, 1942).

Virulence of egg viruses. Burnet (1936 *a*) found that his egg passage M virus was only slightly virulent for ferrets. After intranasal inoculation, although there were no symptoms, the animal became resistant to infection and developed immune bodies (Burnet, 1937 *a*) Egg passage M virus was found to be devoid of pathogenicity for man on intranasal inoculation (Burnet and Lush, 1938 *a*).

Although M egg virus was originally thought to be avirulent for mice, it later appeared that this was not so (Burnet and Lush, 1938 *b*, see also Burnet, 1938). It was found that a proportion of mice inoculated intranasally with this virus died after the 8th day, and might show consolidation of the lungs. It was possible to passage egg virus through mice, when much of the pathogenicity for the egg was lost, and the strain became a normal mouse passage one.

Beveridge and Burnet (1946) report that the properties of the fully adapted Melbourne strain are now as follows it is nonpathogenic for mice and ferrets, it has almost lost the power to agglutinate fowl and pigeon cells, but agglutinates guinea-pig cells, it grows poorly in the allantoic cavity, antigenically it is still close to the mouse-adapted strain, although minor differences are recognizable. The Melbourne Egg strain probably should be regarded as a distinct phase of equal status with O and D

Burnet, Keogh, and Lush (1937) developed a strain EM which produced lesions in the egg while still affecting the mouse This strain was produced from egg passage virus by a few mouse passages, thereafter the strain was passed irregularly both through mice and eggs. This strain could be titrated either on the egg membrane or by its effect on the mouse.

The virulence of WS egg virus for animals has been described by Burnet and Lush (1938 *b*) In contrast to the reactions of M egg virus, WS egg virus has retained some virulence for ferrets, producing mild symptoms and a temperature rise at 48 hours The WS egg virus is also virulent for mice, and resembles the EM strain in that it can be titrated on the egg membrane and in the mouse. Stuart-Harris (1939) has produced a neurotropic variant of WS by egg passage After passage the chick embryos developed hemorrhagic encephalitis, chick embryo brain was then injected intracerebrally in mice, and after a number of passages these animals developed a fatal encephalitis

Swine virus infections This virus can be grown on the chorio-allantois (Kobe and Fertig, 1938) The synergistic effect of *H suis* and swine virus could be reproduced on the chorio-allantois Recently isolated and old stock strains of *H. suis* worked equally well, and even heat-killed cultures could be used, 2 strains of human influenza virus failed to substitute for *H. suis* (Bang, 1943 *a*).

Use of the chorio-allantois in influenza research.

1 Virus can be titrated by pock-counting on the chorio-allantois (Burnet, 1936 *a*, *b*, *c*, Burnet and Faris, 1942)

2 The egg membrane has been found to yield an effective antigen for use in the complement fixation test (Lush and Burnet, 1937, Hoyle and Fairbrother, 1937 *c*)

CHAPTER LIX

CULTIVATION OF INFLUENZA VIRUS

TISSUE CULTURE

VIRUS has been grown in media composed of chick embryo and Tyrode's solution (Francis and Magill, 1935 *a*,[1] 1935–6, 1937 *a*, Smith, 1935; Magill and Francis, 1936, 1936–7, 1937; Francis, 1937, Stokes *et al.*, 1937 *b*, Tulloch, 1939; Eaton, 1940 *a*, Tsurumi *et al.*, 1941 *b*; Andrewes, 1942 *c*; Packaleú, 1946). Cultured virus is said to retain its antigenic properties and its pathogenicity.

Chick embryo lungs, kidney, and spleen can be used as the source of tissue (see Burnet and Clark, 1942).

Virus A has also been cultivated in roller tube cultures using embryonic lung, or the lung of 2-day-old chicks, a higher concentration of virus was found in the tissue portion than in the nutrient fluid, but the titers of both portions remained almost constant from about the 5th–30th days (Enders and Pearson, 1941, Pearson and Enders, 1941).

Cultures prepared by Dochez, Mills, and Kneeland (1934–5, 1936) were said to produce clinical influenza on inoculation into volunteers. In earlier work (1932–3) by these authors, cultures prepared from cases of influenza had produced only common colds on human inoculation.

It has been reported (Francis and Magill, 1937 *c*) that virus may be cultivated directly from filtrates of human throat washings by inoculation of tissue cultures

Tissue cultures have also been used to study the effect of antiserum and virus on cells (see p. 648), to immunize human beings (see p. 658), and to serve as antigen in complement fixation tests (see p. 649).

The Interference Phenomenon

Andrewes (1942 *a*) has studied the interference phenomenon in tissue cultures He cultivated virus A, and added a different strain of the same type 24 hours later. The first virus interfered with, and prevented the growth of, the second. This interfering effect of the first strain did not prevent multiplication of LGI virus added later. The strain of virus added first, interfered with the growth of the second If the two strains were added simultaneously the one present in the larger amount suppressed the growth of the other

Lennette and Koprowski (1946) made the interesting observation that a tissue culture strain of yellow fever (17 DD High) and the West Nile virus suppressed and interfered with the growth of PR8 in tissue culture

THE EGG

Practically all work has been carried out with hen eggs, but duck eggs can be used for certain purposes (Beveridge and Burnet, 1946)

The Chorio-Allantoic Membrane

Influenza virus A can be cultivated on the chorio-allantoic membrane (Smith, 1935, Burnet, 1935 *b*, 1936 *a*, *b*, *c*, *d*, 1937 *a*, Badenski and Bruckner, 1937, Hoyle and Fairbrother, 1937 *c*, Tsurumi, Ogasawara, and Takagi, 1939)

Adaptation to the chorio-allantois. It is not easy to adapt strains fully to the chorio-allantois, and only the Melbourne and WS strains have been so adapted Certain other strains produce foci, but do not kill the embryo (Beveridge and Burnet, 1946). Burnet (1936 *a*) has made a particular study of the effect of his

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

. . . ds definite isolated
. . . was killed on the
. . . ed a hemorrhagic
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2. The egg membrane has been found to yield an effective antigen for use in the complement fixation test (Lush and Burnet, 1937, Hoyle and Fairbrother, 1937 *c*).

3. Virus grown on the egg can be used to immunize animals (Burnet, 1937 *a*, 1938), and man (Burnet and Lush, 1938 *a*).

4. The egg can be used, it has been reported, for the direct isolation of virus, without preliminary animal passage, from nasopharyngeal washings (Francis and Magill, 1937 *c*; Hyde and Chapman, 1937).

5. The egg can be used as an indicator of virus neutralizing properties. The virus and serum under test are mixed and inoculated in the egg. The capacity of the serum for preventing the development of pocks is noted, and forms an estimate of its immune body content (Burnet, 1936 *a*, Burnet and Lush, 1938 *a*).

6. The egg has been used extensively to study the academic problem of the antigen-antibody reaction in influenza (Burnet, 1936 *d*; Burnet, Keogh, and Lush, 1937).

Allantoic Inoculation

Influenza virus proliferates readily in the allantoic membrane, and is found in large amount in the allantoic cavity; this route of inoculation is widely used in influenza work (Burnet, 1941 *c*, 1942 *b*; Henle and Chambers, 1941; Hirst, 1941, Nigg, Wilson, and Crowley, 1941, Eaton *et al*, 1942; Beveridge and Burnet, 1946).

Both types of human virus and swine virus grow well in the allantoic cavity, with the proviso that freshly isolated strains in Burnet's O phase may only do so [illegible] (see Ch. LVI).

[illegible] that influenza virus A and B will proliferate in [illegible] ly infected in the yolk sac with typhus *Rickettsiae* (Breinl and Castaneda strains)

Preliminary incubation. It is customary to inoculate eggs already incubated for 10 days at about 38–39° C., but Beveridge (1944 *a*) found that embryos incubated at 36.6 to 37.8° C. give about twice as much allantoic fluid as those incubated at 39° C., and infection was secured more readily. If older eggs are used, trouble is experienced with the heavy precipitate of urates.

Inoculum As mentioned earlier (see p. 583) virus can be isolated by inoculation of throat washings, with or without preliminary treatment to reduce contaminants, directly into the allantoic cavity, in which case a volume of 0.15 to 0.2 c.c. is used

When passage strains are inoculated, it is best to use weak dilutions, 10^{-3} to 10^{-5}, as interference may result with more concentrated inocula (see p 621) Volumes of 0.05 c.c. to 0.2 c.c. are usually used. Whether or not infection occurs, depends on the amount of virus introduced into the allantoic fluid, and not on the volume of the inoculum (Taylor et al., 1944 *a*).

Temperature after inoculation Better virus yields are obtained when the eggs are incubated after inoculation at 35–36° C rather than higher temperatures (Miller, 1944 *a*, McLean *et al*, 1944, 1945 *a*, Sigurdsson, 1944) The more acid pH at higher temperatures may be the explanation of this phenomenon

It is usual to incubate for 40–48 hours

Evidences of infection The presence of infectivity is most easily judged by the occurrence of agglutination when allantoic fluid is mixed with a chick red cell suspension Allantoic fluid usually gives a titer of 1 256, 1 512 or 1/1024 with 1.5 or 2 per cent chick cells A high titer may be found even after 12 hours (Henle and Chambers, 1941) The fluid of eggs inoculated with untreated washings may cause a nonspecific agglutination of fowl cells, up to 1 160, due to the presence of cocci or *B subtilis* (Florman, 1946) Infectivity can also be tested by mouse inoculation or inoculation into a fresh series of allantoic cavities Infectivity is maximal after 48 hours incubation and may reach 10^{7} (Burnet, 1941 *c*) The concentration of virus particles in untreated allantoic fluid has been calculated at 0.01 per cent (Friedewald and Pickels, 1944)

The injection of undiluted infected allantoic fluid, irradiated for 3–5 minutes,

in 8-day embryos, did not prove lethal, but the allantoic sacs frequently failed to develop further, when fluids were irradiated for 15 minutes or more, no inhibition of growth of the sacs was produced, nor did the fluids act as interfering agents (see below), however, virus particles were adsorbed onto allantoic cells regardless of the time of exposure to UVL (Henle and Henle, 1947)

The presence of virus can be shown indirectly by using the fluid as an antigen with known sera in neutralization, or complement fixation tests The peak of complement fixing activity occurs after 36 hours, and that of flocculability after 48 hours (Henle and Chambers, 1941).

The pH values of allantoic fluids from noninfected eggs show a rapid drop from the 12th–17th day of development In contrast, the allantoic fluid of influenza-infected eggs gives pH values remaining relatively stable about the neutral point between 11 and 17 days, the Eh of normal fluid usually shows a positive potential, but there is a marked state of reduction accompanying virus growth, with a predominantly negative potential (*U.S Nav Med Lab.* No 1, 1945)

Embryos are seldom killed after allantoic inoculation, but the chorio-allantois adapted Melbourne and WS strains usually kill within 48 hours, with widespread hemorrhages in muscles and brain (Beveridge and Burnet, 1946).

Distribution of virus After allantoic inoculation, virus reaches all parts of the egg, including the amniotic fluid, embryo, and even the yolk sac (see Pearson, 1944 *a*). Virus grows in the allantoic entoderm and the cells may show degenerative changes (Burnet, 1941 *c*).

Risk of contamination. The healthy embryo is relatively resistant to infection with bacteria, and contamination during routine passage experiments is unusual. It appears, however, that with the swine strain there is an increased susceptibility to infection (Taylor *et al.*, 1945).

Titration by allantoic inoculation Influenza virus preparations can be titrated by the inoculation of falling dilutions in a series of allantoic cavities. The presence of infectivity is judged by the agglutination of red cells, 50 per cent. end-points can be worked out by Reed and Muench's (1938) method. For example, Hirst (1942 *a*) injects serial tenfold dilutions of the virus, made in horse-serum broth, into the allantoic cavity, 6 eggs being used for each dilution, and incubated at 37° C. for 42 hours After chilling at 4° C for 3–4 hours, the allantoic fluid is aspirated from the air sac end The presence of virus is detected by mixing equal (e g, 1 c c.) quantities of pure allantoic fluid and chick cell suspension, and examining for agglutination after an hour. This method proves more sensitive than the inoculation of mice, and is equally useful for mouse-adapted and recently isolated strains

Knight (1944 *b*) found that high degrees of accuracy are obtained with 10 embryos, enabling twofold differences to be recognized, but that sufficient accuracy results from the use of half that number (recognizing fourfold differences). He also confirmed that the embryo is sensitive to considerably smaller amounts of virus than can cause infection of mice.

Uses of allantoic inoculation.

1 For the isolation and characterization of strains from throat washings (see p 591)

2 For passage of strains in the laboratory.

3 For titration experiments (see above)

4 For obtaining large quantities of crude virus that can be purified to study its biophysical and biochemical properties (see Ch LVIII).

5 Allantoic fluid is the commonest source of virus for use in influenza vaccine (see Ch LXIII)

6 Allantoic fluid can be used as an antigen in complement fixation tests (see p 649), or in virus neutralization tests (see p. 647).

7. For studying problems such as the interference phenomenon (see p. 621), or the neutralization of virus by antisera.

8. For chemotherapeutic experiments (see p. 627).

Amniotic Inoculation

Inoculation of influenza virus into the amniotic cavity was introduced by Burnet (1940 *a*, *b*). Burnet, his colleagues, and others have since used the method extensively (see, e.g., Burnet, 1941 *a*, 1942 *a*, Burnet and Foley, 1940, 1941 *b*, *c*; Burnet *et al.*, 1942, Eaton *et al.*, 1942; Taylor and Chialvo, 1942; Burnet, Beveridge, and Bull, 1944; Burnet and Stone, 1945 *b*, *c*, Beveridge and Burnet, 1946, Burnet, Stone, and Anderson, 1946).

The technique can be used for the primary isolation of strains from throat or nasal washings, as well as for passage of laboratory strains. Special precautions are necessary to prevent O phase virus entering the D phase (see p. 595). Various methods have been introduced for the actual inoculation, and these are fully described in Ch. XIII

Incubation and inoculation. It is usual to use eggs incubated for 13–15 days before inoculation. The temperature of incubation before inoculation has no influence on the susceptibility to virus A or B (Beveridge, 1944 *a*). Burnet, Beveridge, and Bull (1944) found that 8–10-day embryos were more sensitive in detecting limiting dilutions of an unadapted B strain (Bon), but a number of B strains isolated in 1945 infected 13-day embryos (Beveridge and Burnet, 1946). As regards the inoculum, this should be as dilute as possible, especially when it is desired to maintain virus in the O phase. Eggs inoculated with virus A should be examined after 48–72 hours, but those inoculated with virus B should be left for 5 days, owing to the slower development of the virus.

Distribution of virus after amniotic inoculation. Virus can be detected in the allantoic fluid as well as the lungs, tracheal fluid, and amniotic fluid It appears as if virus inoculated into the amnion is swallowed, some reaching the trachea where proliferation occurs. Virus from the trachea is swallowed and passes through the alimentary canal to reach the allantoic cavity (Burnet, 1940 *b*). Virus probably proliferates in the pharynx also.

Evidence of infection. Both amniotic and allantoic fluids contain virus, and give agglutination of chick red cells The embryo shows striking changes, and the following description is largely taken from that of Burnet (1940 *a*, *b*). The changes are similar with O and D phase (Beveridge and Burnet, 1946) We have ourselves confirmed these changes, and paid particular attention to the histological appearances

The embryo is usually underdeveloped and after 3 days many will be moribund or actually dead The tracheal fluid should be removed, centrifuged, and films of the deposit stained by Leishman's method (see Ch XIII for technique involved). This fluid is turbid and contains granular clumps Microscopic examination shows the presence of vacuolated epithelial cells (12–20 μ in diameter), deriving chiefly from the parabronchi, also leukocytes, and immature red cells with disintegrated nuclei. The vacuolated cells may contain granular eosinophilic material With a large inoculum, the tracheal fluid may show these appearances after 24 hours, and will certainly do so after 48 hours, with unadapted strains, changes may not be evident for 4–6 days, and even then be only slight.

The lungs appear to be whiter than normal, and more firm The main changes are to be found histologically, and are similar with viruses A and B (Burnet, 1940 *a*, *b*, 1942 *a*, Fodden and Rhodes) The earliest lesions occur in the parabronchi, with involvement of the epithelial cells The nuclei show signs of degeneration, later there is much cellular proliferation, and the epithelium desquamates, to fill the lumen. The saccular outgrowths from the parabronchi become disorganized,

and so does all the lung tissue. The endothelial lining of the blood vessels surrounding the parabronchi proliferates. Numerous leukocytes are found at a late stage.

Titration of viruses by the amniotic route. Viruses can be titrated by amniotic inoculation, and the method is as sensitive as mouse inoculation (Burnet, 1941 *a*). Fifty per cent. end-points can be calculated, evidence of infection being indicated by the occurrence of chick cell agglutination with the amniotic fluid.

The effect of passage. Unless special precautions are taken, as described on p 595, virus passed by the amniotic route will enter the D phase. As regards virulence, Burnet and Foley (1941 *b*) studied the changes in activity of 2 strains (Bundoor and R). They found a rapid increase in virulence for the embryo, with a shortened survival period The power to produce signs in ferrets progressively diminished, but the immunizing potency was unaltered

Uses of amniotic inoculation.

1. For the isolation of strains from human material (see p. 583).
2. For passage of strains in the laboratory.
3. For titration experiments
4. For studying academic problems such as the pathological effects produced by influenza virus, or the neutralization of virus by antisera.

Inoculation by Other Routes

Virus can be isolated from washings by inoculation of the yolk sac of 3–6 day eggs (Beveridge, Burnet, and Williams, 1944), but about 100 times as much virus is needed as will infect amniotically (Beveridge and Burnet, 1946), eggs can be inoculated with filtrates or with raw washings using penicillin and sulfadiazine. Passage strains of influenza A and B can be propagated by this route if desired (Beveridge, 1944 *a;* Burnet, Beveridge, and Bull, 1944). Using a modified technique of yolk sac inoculation, Nigg, Crowley, and Wilson (1940) found that suspensions of yolk sac, chorio-allantois, and amnion yielded excellent antigens for complement fixation

The Melbourne Egg strain infects embryos on intravenous inoculation, producing characteristic hemorrhagic lesions (Burnet and Bull, 1944).

The Interference Phenomenon in Eggs

The interference phenomenon has been studied in eggs, as well as in tissue cultures (see above). The fundamental observations are largely those of Henle and Henle As a rule, virus has been inoculated in the allantoic cavity, and subsequent tests for the presence of virus conducted by means of chick cell agglutination.

I. Interference produced by concentrated single inocula.

Following a single inoculation of virus in the allantoic cavity, some particles may become inactivated, and interfere with the propagation of the remaining viable particles (Henle and Henle, 1943) Thus, if the inoculum used for allantoic inoculation is too concentrated, especially with the Lee (B) strain, the fluid removed after incubation will not give such a high titer in chick cell agglutination tests as would be obtained by the inoculation of more dilute material (Henle and Henle, 1944 *a*). Similarly a greater degree of toxicity is developed in allantoic fluid by inoculation of dilute "seed" (Henle and Henle, 1946 *b*).

When a single inoculum is heated, e g, to 56° C. for 10 minutes, partial destruction of virus takes place. This inactive virus interferes with the growth of the remaining active virus in the same preparation, if the heated material is inoculated "neat" in the allantoic cavity (Henle and Henle, 1944 *a*, Jones, 1945). However, if the heated material is inoculated in a dilution of 10^{-2}–10^{-3}, virus growth takes place more readily (Henle and Henle, 1944 *a*) Similar results were obtained with virus partly inactivated by ultraviolet light

By continued transfer of undiluted inocula Magnus (1946) claims to have produced a variant of PR8 that interferes with the propagation of virus (see also Gard and Magnus, 1946).

2. Interfering effect of one dose of virus upon another.

Under certain conditions, the injection of one strain of virus in the allantoic cavit[illegible] another strain Ziegler and [illegible] ound that the inoculation [illegible] inoculated later. They found that interference is completely reciprocal, if small doses are used for the first as well as second inoculum, small doses of Lee are, however, less effective in preventing large doses of PR8 from growing than the reverse. They also found that the interfering effect depends on the relative dosage of the 2 inocula, and the interval between inoculations. The phenomenon can be reversed; for example, if a large second dose of Lee virus is given up to 8 hours after a first small dose of PR8, then PR8 is inhibited. The interfering factor can be destroyed at 56° C., but resists storage at 4° C. for prolonged periods.

3. Interfering effect of irradiated virus.

(*a*) Most work on the interfering effect has been carried out with material inactivated by exposure to ultraviolet light (UVL). When allantoic fluid is irradiated, the infectivity decreases rapidly, the fluid becomes noninfective when the interfering capacity is reaching its peak. On further irradiation, the interfering property diminishes, and finally disappears, although the hemagglutinating capacity of the preparation is still intact (Henle and Henle, 1945 *d*, 1947).

(*b*) Virus almost completely inactivated by irradiation with UVL interferes with the growth of active virus inoculated 3–4 hours later. Dialyzed fluid is more sensitive to irradiation, and exerts the interfering effect only if irradiated for a much shorter period (Henle and Henle, 1944 *a*, *b*, 1947).

Ziegler, Lavin, and Horsfall (1944) found that the inoculation of highly concentrated preparations of A or B virus interferes with the multiplication of homologous or heterologous virus, inoculated 24 hours later They found a similar interference using virus completely inactivated, although inactive PR8 did not produce marked interference with swine virus

Heterologous interference takes place equally well as homologous. Thus irradiated PR8 prevents the multiplication of active PR8, Lee, or WS injected later (Henle and Henle, 1945 *c*)

(*c*) The dose of irradiated fluid required to interfere is very small, and 0.01 to 0.005 c.c. suppresses the formation of measurable amounts of hemagglutinin from a subsequent inoculation of active virus High concentration of test virus does not overcome this interfering effect (Henle and Henle, 1944 *b*).

(*d*) The factor in UVL-irradiated virus remains active when stored at 4° C for 4 weeks (Ziegler, Lavin, and Horsfall, 1944)

(*e*) There is no striking difference in the interfering property of UVL-irradiated fluid harvested after 24 to 96 hours, although their agglutinating titers vary, the interfering property in fluids harvested at the earlier periods is more susceptible to destruction by UVL (Henle and Henle, 1944 *b*)

(*f*) With regard to the time factor, the interfering injection of UVL-irradiated virus can be given as early as 96 hours before the test virus, or up to 3 hours later (Henle and Henle, 1944 *b*)

4. The site of interference.

The interfering injection appears to act on the cells of the allantoic cavity, and renders them insusceptible to attack by active test virus If the allantoic sac is flushed with saline after the inoculation of the interfering agent, interference still

occurs The protection of susceptible cells takes place so rapidly that even if virus is introduced during the process of flushing, interference still results (Henle and Henle, 1944 *b*).

5. Interference between unrelated viruses.

Irradiated influenza virus interferes with the subsequent growth of western *equine encephalomyelitis virus, and epidemic keratoconjunctivitis virus (Henle and* Henle, 1945 *c*). This need only indicate that both viruses react with the same cell receptors.

6. The nature and mode of action of the interfering agent.

Interference does not result in destruction of virus, and some active virus can be found in fluids when interference has taken place This virus may derive from the interfering injection, if it contains some active virus, or from the test virus which retains its original activity although it cannot proliferate (Henle and Henle, 1944 *b*).

The interfering agent in irradiated preparations appears to be the inactivated virus itself for the following reasons It is sedimented in the centrifuge under conditions that will sediment the active virus It is adsorbed on to, and eluted from, chick red cells in amounts comparable to the virus It is specifically neutralized by immune sera, the interfering agent in virus A preparations is neutralized only by antisera to virus A (Henle and Henle, 1945 *d*).

The interfering injection prevents the test virus from entering the cells and propagating in the allantoic sac; active virus cannot be detected in suspensions of embryonic membranes (Henle and Henle, 1944 *b*).

It is uncertain whether irradiated preparations contain a small amount of active virus or not From the nature of the phenomenon it is difficult to demonstrate the presence of virus in a preparation that effectively prevents its proliferation (Henle and Henle, 1944 *b*) It would appear that for all practical purposes it can be considered that completely inactivated virus acts as an interfering agent. The interfering agent may produce its effect by saturation of receptors on susceptible cells, so that the subsequently introduced test virus cannot "attach" itself and grow, it is possible that the reaction may occur intracellularly and involve enzyme systems (Ziegler, Lavin, and Horsfall, 1944, Henle and Henle, 1945 *c*)

In later work, Henle and Henle (1947) showed that virus particles irradiated for too long (1–2 hours) to permit them to give the interfering effect were still adsorbed to the allantoic cells If the virus had not been irradiated for more than 3–5 minutes, this adsorption resulted in cessation of growth of the allantoic sac, and at the same time the interference phenomenon occurred These observations

(1) adsorption, (2) an alteration occurs in the function of cell, which excludes other viruses, this is perhaps initiated by the entry of a single particle; (3) intracellular proliferation, (4) release of virus

7. The interference phenomenon to demonstrate growth cycles.

Henle, Henle, and Rosenberg (1947) adapted the interference phenomenon to study the growth of virus in the allantoic sac, and found that multiplication is apparently based on general principles similar to those affecting the propagation of bacterial viruses In this work, the PR8 and Lee strains of virus were used.

1 Injection of 1000–10,000 ID 50 of virus in the allantoic sac did not lead to complete adsorption onto the cells of the sac, but between 10 and 30 per cent. of *virus remained free. In the case of PR8, 6 hours, in the case of Lee,* 9–10 hours

By continued transfer of undiluted inocula Magnus (1946) claims to have produced a variant of PR8 that interferes with the propagation of virus (see also Gard and Magnus, 1946).

2. Interfering effect of one dose of virus upon another.

Under certain conditions, the injection of one strain of virus in the allantoic cavity inhibits the multiplication of a subsequent inoculum of another strain. Ziegler and Horsfall (1944) used PR8, Lee, and swine strains, and found that the inoculation of any one strain inhibits the growth of another strain inoculated later. They found that interference is completely reciprocal, if small doses are used for the first as well as second inoculum; small doses of Lee are, however, less effective in preventing large doses of PR8 from growing than the reverse. They also found that the interfering effect depends on the relative dosage of the 2 inocula, and the interval between inoculations The phenomenon can be reversed, for example, if a large second dose of Lee virus is given up to 8 hours after a first small dose of PR8, then PR8 is inhibited The interfering factor can be destroyed at 56° C., but resists storage at 4° C. for prolonged periods.

3. Interfering effect of irradiated virus.

(*a*) Most work on the interfering effect has been carried out with material inactivated by exposure to ultraviolet light (UVL). When allantoic fluid is irradiated, the infectivity decreases rapidly, the fluid becomes noninfective when the interfering capacity is reaching its peak. On further irradiation, the interfering property diminishes, and finally disappears, although the hemagglutinating capacity of the preparation is still intact (Henle and Henle, 1945 *d*, 1947).

(*b*) Virus almost completely inactivated by irradiation with UVL interferes with the growth of active virus inoculated 3–4 hours later. Dialyzed fluid is more sensitive to irradiation, and exerts the interfering effect only if irradiated for a much shorter period (Henle and Henle, 1944 *a*, *b*, 1947).

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(*d*) The factor in UVL-irradiated virus remains active when stored at 4° C. for 4 weeks (Ziegler, Lavin, and Horsfall, 1944)

(*e*) There is no striking difference in the interfering property of UVL-irradiated fluid harvested after 24 to 96 hours, although their agglutinating titers vary, the interfering property in fluids harvested at the earlier periods is more susceptible to destruction by UVL (Henle and Henle, 1944 *b*)

(*f*) With regard to the time factor, the interfering injection of UVL-irradiated virus can be given as early as 96 hours before the test virus, or up to 3 hours later (Henle and Henle, 1944 *b*).

4. The site of interference.

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CHAPTER LX

REACTION OF INFLUENZA VIRUS TO PHYSICAL, CHEMICAL, AND BIOLOGICAL AGENTS

PHYSICAL AGENTS

Heat

THE INFECTIVITY of virus is destroyed at 56° C in about 20–30 minutes, although the interfering property and chick cell agglutinating property resist. Virus strains can be "trained" to withstand temperatures normally lethal (Jones, 1945).[1]

Storage

Preparations of crude or purified influenza virus (human and swine) derived from mouse lungs or egg fluids can be kept for at least several weeks if desiccated *in vacuo*, preferably from the frozen state, and kept in the refrigerator (Scherp *et al*, 1938, Shope, 1931 *b*, 1934 *b*; Hare, McClelland, and Morgan, 1942) Ferret turbinates can be stored in 50 per cent. glycerol in Ringer for at least a year at 0° C (Burnet and Clark, 1942) We have found that virus in infected allantoic fluid, kept in the ordinary cold room, retains sufficient activity to infect eggs for at least 6 weeks

The most satisfactory method of storage (e g, of allantoic fluid or mouse lung suspension) is in ampules in dry ice at about –60° C. (see, e g, Turner, 1938, Horsfall, 1939, Hirst, 1941). Virus can be maintained easily in amniotic fluid with 30 per cent. glycerol at –10° C. (Burnet and Stone, 1946). Apparently, certain B strains deteriorate rapidly on freezing (Hirst, 1947 *b*).

Simple Desiccation

When infective allantoic fluid of high potency is dried in air on glass it may remain viable and infective for eggs for several days, when mucus is added, the survival time is prolonged for about 7 weeks (Parker, Dunham, and MacNeal, 1944). Virus B was found to be inactivated more quickly than virus A when dried on glass or cellophane (*U.S Nav Med. Lab*, 1942 *b*).

Survival in Fabrics and Dust

Virus can survive when impregnated in a blanket and dried, and it can be distributed in the air or dust by shaking (Edward, 1941).

A small percentage of virus survives when impregnated in fabric and dried in air, in the dark, some virus survives for weeks, but it disappears more rapidly if the materials are kept at 37° C, or in the light (Edward, 1941). Virus has been demonstrated in dust exposed near an infected ferret (Edward, 1941).

Sonic Vibration and Homogenization

Virus A and swine virus are not destroyed by intense sonic vibration, nor does homogenization increase the activity of infected tissue (Scherp and Chambers, 1936–7). As described above (see p 588), sonic vibration results in the partial disintegration of elementary bodies with the release of some soluble substance.

Irradiation

X-ray therapy given to mice infected with swine virus had no effect, therapy administered 48 hours before infection produced a decreased mortality (Dubin, Baylin, and Gobbel, 1946).

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

elapsed before the concentration of virus began to rise in the fluid. It appeared that during this time virus increased in association with the cells.

2. To obtain "single infection" of cells, small doses of active virus were injected at the onset of the experiments. Then to interrupt the process of adsorption, and to prevent further infection of cells, irradiated heterologous virus was injected shortly after. This virus was shown to produce the interference phenomenon in all remaining susceptible cells, and to prevent further adsorption of virus

The content of virus in the allantoic fluid remained constant for 6–9 hours, then rose steeply for 2 hours (PR8) or 2–3 hours (Lee) From this period on, the active virus titer remained on this higher "plateau" up to 24 hours after injection, when the experiment was ended. By this technique, "one-step growth curves" were thus obtained, closely resembling those of bacterial viruses.

3. Varying the concentration of active virus in the inoculum over a wide range did not result in significant alterations in the one-step curve

4. Calculations showed that for every one ID 50 of virus adsorbed, about 50 were released. It is not known how many virus particles make up one ID 50

5. When homologous irradiated virus was used, the yield of virus was reduced to a considerable extent. The "step" was delayed, and decreased in size. This effect was linked to the virus particle, and was destroyed by prolonged irradiation

6 No information is available as to what happens within the cells during the few hours before virus is liberated. It appears most likely that the virus yielded into the allantoic fluid is released by the destruction of the entodermal cells of the allantoic sac, and histological observations carried out on the allantoic cells seemed to be in agreement with this view.

TABLE 26

SUBSTANCES INACTIVATING INFLUENZA VIRUS

Phenol, 0 5 per cent–3 per cent	Dunham and MacNeal (1944)
Iodine, chlorine, or bromine	Dunham and MacNeal, Knight and Stanley (1944), Stone and Burnet (1945)
Lugol's solution, 1 per cent	Dunham and MacNeal
Mercuric chloride, 1/1,000	Dunham and MacNeal
Potassium permanganate, 1/1,000	Dunham and MacNeal
Copper sulphate, 1 per cent	Dunham and MacNeal
Propylene glycol, 90 per cent	Dunham and MacNeal
Liquor antisepticus (N F.VII) 80 per cent	Dunham and MacNeal
Salts of heavy metals	Knight and Stanley
Mercurochrome	Knight and Stanley
Ascorbic acid	Klein (1945)
Ether (10 per cent is withstood for at least 90 hours)	Sarracino and Soule (1941 *b*)
Bile salts	Smith (1939)
Sodium desoxycholate, saponin	Burnet, Lush, and Jackson (1939)
Sodium lauryl sulfate	Burnet and Lush (1940 *b*)

SUBSTANCES FAILING TO INACTIVATE INFLUENZA VIRUS

Reducing agents	Knight and Stanley (1944)
Glucose	Knight and Stanley
Ammonium sulfate	Knight and Stanley
Calcium chloride	Knight and Stanley
Sodium thiosulfate, arginine	Knight and Stanley
Trypsin and chymotrypsin	Merrill (1936)

Soaps and Detergents

Stock and Francis (1940, 1943) found that a number of virus strains were effectively inactivated at pH 7 5 by oleic, linolic, and linolenic acids

Detergents of various types have also

(Stock and Francis, 1940, Klein and Stev

however, think these agents to be suffic

ivory soap in a 1 per cent solution killed virus, but 15 minutes or more was needed

Soap solution quickly destroys virus spread on the palm of the hand (Parker and MacNeal, 1944).

CHEMOTHERAPY

The following agents also have been found devoid of protective effect *in vivo*. acridines, guanyl compounds, alkaloids, arsenicals, antimonials, gold compounds, oils, salicylates, peroxides, formaldehyde compounds, nitro compounds, dyes, and miscellaneous chemicals (Andrewes, King, and van den Ende, 1943, see also *U.S. Nav Med Lab*, 1943 *a*, Cutting *et al*, 1947).

Quinine administered in the diet had a slight but consistent retarding effect on the course of infection in mice (Seeler, Graessler, and Ott, 1946).

Various antibiotics have been found valueless in the treatment of experimental influenzal infection penicillin, tyrothricin, tyrocidin, gramicidin, streptomycin (*U.S Nav. Med Lab 1943 a, Florman, Weiss, and Council, 1046, Cutting et al*, 1947)

Streptomycin appears to have no inhibitory effect on the growth of virus A and B in the allantoic sac (Lowell and Buckinham, 1946).

Ultraviolet irradiation has been extensively studied. With the lamp used virus in allantoic fluid withstood about 60 minutes exposure, but after dialysis, infectivity was lost in about 30 seconds (Henle and Henle, 1944*a*, 1947). McLean *et al* (1945 *d*) investigated the reaction of purified swine virus. Tables showing the rate of fall in titer in irradiated specimens are available (Henle and Henle, 1946*b*). A wavelength of about 2650 Å appears to be the most destructive (Hollaender and Oliphant, 1944).

The effect of irradiation on the various properties of influenza virus will be found in the appropriate sections. In this connection, Henle and Henle (1947) have carried out a detailed study, and have shown that the biological properties are lost in the following order (1) Ability to propagate (2) Toxicity. (3) Interfering property in the chick embryo, and the inhibitory effect on embryonic development. (4) Hemagglutinating capacity, including adsorption-elution, the ability to render red cells refractory to agglutination by fresh virus, and adsorption on to cells of the allantoic sac. (5) CF activity is partly destroyed together with the HA (600 S component), and is partly more resistant (possibly 30 S).

They have shown that 4 distinct preparations can be obtained. (1) After irradiation for 1–2 hours, the preparation agglutinates red cells and renders them inagglutinable This preparation is adsorbed onto cells of the allantoic sac, but causes no visible changes. (2) After irradiation for 3–5 minutes, the fluid behaves similarly, but in addition alters the cells of the allantoic sac, so that they become insusceptible to infection by active virus (3) After exposure for ½–1 minute, the preparation is, in addition, toxic. (4) Nonirradiated virus multiplies in the allantoic sac.

SURVIVAL AND DESTRUCTION OF ATOMIZED VIRUS IN AIR

A number of experiments has been carried out in which virus has been atomized in the form of a mist in special chambers, or even small rooms, and its survival tested by exposing mice, these animals can be infected readily by inhalation Thus Wells and Brown (1936) found that virus could be recovered from air after 30 minutes

Virus survived much longer in dry atmospheres, and at relative humidity 17–24 per cent it remained infective for 24 hours (Loosli *et al*, 1943) A dried virus mist was found to be more resistant to the destructive action of iodine vapor than a moister one (Stone and Burnet, 1945)

Virus suspended in air in the form of atomized droplets can be destroyed (so as not to infect exposed mice) by ultraviolet light (Wells and Brown, 1936, Wells and Henle, 1941, Stokes and Henle, 1942) Edward, Lush, and Bourdillon (1943) found light of wavelength 2537 Å highly effective

The atomization of various antiseptic substances also destroys infective droplets of influenza virus propylene glycol (Henle and Zellat, 1941, Stokes and Henle, 1942), iodine (0 1 p p m, Stone and Burnet, 1945). Sodium hypochlorite (1 per cent) destroyed 90 per cent of atomized virus A in a quarter of an hour, and hypochlorous acid gas, 1 volume in 2 million volumes of air, was probably even more effective (Edward and Lidwell, 1943)

The infectivity of virus A was completely destroyed by exposure to the vapors of α or β naphthyl isocyanate, phenyl isocyanate, and p-nitrobenzoyl chloride, oxyquinoline, thio-urea, oil of nutmeg, and oil of mustard showed slight activity (Grubb, Miesse, and Puetzer, 1947).

CHEMICAL AGENTS

A considerable number of chemical substances has been tested for their inactivating effect on virus *in vitro* Formalin is perhaps the most widely used In a strength of 0 08 per cent, virus is destroyed in 8 days (McLean *et al*, 1945 *d*)

The following tables show the results of tests with numerous other substances

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Liquor antisepticus (N F.VII) 80 per cent	Dunham and MacNeal
Salts of heavy metals	Knight and Stanley
Mercurochrome	Knight and Stanley
Ascorbic acid	Klein (1945)
Ether (10 per cent is withstood for at least 90 hours)	Sarracino and Soule (1941 *b*)
Bile salts	Smith (1939)
Sodium desoxycholate, saponin	Burnet, Lush, and Jackson (1939)
Sodium lauryl sulfate	Burnet and Lush (1940 *b*)

SUBSTANCES FAILING TO INACTIVATE INFLUENZA VIRUS

Reducing agents	Knight and Stanley (1944)
Glucose	Knight and Stanley
Ammonium sulfate	Knight and Stanley
Calcium chloride	Knight and Stanley
Sodium thiosulfate, arginine	Knight and Stanley
Trypsin and chymotrypsin	Merrill (1936)

Soaps and Detergents

Stock and Francis (1940, 1943) found that a number of virus strains were effectively inactivated at pH 7 5 by oleic, linolic, and linolenic acids

Detergents of various types have also been [illegible]
(Stock and Francis, 1940, Klein and Stevens,
however, think these agents to be sufficientl
ivory soap in a 1 per cent solution killed virus, but 15 minutes or more was needed

Soap solution quickly destroys virus spread on the palm of the hand (Parker and MacNeal, 1944).

CHEMOTHERAPY

Despite a preliminary suggestion that sulfonamides might have some beneficial effect on mice infected with influenza (Climenko, Crossley, and Northey, 1939), it is not now believed that they have any protective action (Coggeshall and Maier, 1942, Andrewes, King, and van den Ende, 1943, *U.S Nav. Med. Lab*, 1943 *a*; Harford, Smith, and Wood, 1946, Cutting *et al*, 1947).

The following agents also have been found devoid of protective effect *in vivo*· acridines, guanyl compounds, alkaloids, arsenicals, antimonials, gold compounds, oils, salicylates, peroxides, formaldehyde compounds, nitro compounds, dyes, and miscellaneous chemicals (Andrewes, King, and van den Ende, 1943, see also *U.S Nav Med Lab.*, 1943 *a*, Cutting *et al*, 1947).

Quinine administered in the diet had a slight but consistent retarding effect on the course of infection in mice (Seeler, Graessler, and Ott, 1946).

Various antibiotics have been found valueless in the treatment of experimental influenzal infection penicillin, tyrothricin, tyrocidin, gramicidin, streptomycin (*U S Nav Med Lab* 1943 *a*, Florman, Weiss, and Council, 1946, Cutting *et al*, 1947).

Streptomycin appears to have no inhibitory effect on the growth of virus A and B in the allantoic sac (Lowell and Buckinham, 1946)

It has been reported that nitroakridin 3582 has an inhibitory effect on the growth of influenza virus B in the allantoic sac (Green, Rusmussen, and Smadel, 1946), and McClelland has confirmed this point in these laboratories; hexamidine (4,4′ diamidino diphenoxy hexane di-isethionate) has a similar effect (McClelland and van Rooyen, 1948).

Apple pectin injected in the allantoic sac shortly before or after virus was found to inhibit virus multiplication, gum acacia showed a similar but less pronounced action (Green and Wooley, 1947).

Rubin and Giarman (1947) showed that various antibiotic lactones and analo-
...butyrolactone; 6-methoxy-8-(2,5-
...4-olide; 3-methyl-5-carboxy-2-

INFLUENCE OF BUFFERS AND pH

Knight (1944 *a*) investigated the effect of various buffers on the stability of centrifugally purified PR8 virus at pH 7. Borate, veronal, and phosphate buffers, at a concentration of about 0.1 M, maintained activity at 4° C. for several weeks. The phosphate buffer is probably the most practical. Unbuffered saline was found to be a poor solvent for preserving activity, irrespective of pH.

Miller (1944 *b*) has investigated the influence of pH on the infectivity of various virus strains at a protein concentration of 0.1 mgm. per c.c. in phosphate buffer. Infectivity of PR8 was most stable at pH 6.5–7, swine virus at pH 7–7.9, and Lee virus at 7.9 or higher. Under suitable conditions of buffer and pH, infectivity decreased while chick cell agglutinating activity remained unaltered; the rate of loss of infectivity was greater than the
When tests of stability were performed in a
strains showed less marked differences, and
agglutinating activity and infectivity than when stored at the same concentration in phosphate buffer. Under these conditions, the Lee and swine strains possessed maximum stabilities of agglutinating activity and infectivity at pH 7–8, while the infectivity of PR8 was more stable at pH 7 than 8. In detailed experiments with Lee virus it was found that infectivity and agglutinating activity were maintained best in the composite phosphate-glycine-NaCl buffer.

With PR8, activity was found to be much more stable at 4° C. than 23° C. When stored at concentrations of 2 mgm. per c.c. at 4° C. in phosphate buffer at pH7, the PR8 and Lee strains were much more stable than when stored at 0.1 mgm. per c.c. There was no significant loss over 2 months.

BIOLOGICAL AGENCIES

The Skin

Estimates of the time in which virus A and B is inactivated on the human skin vary from 10 minutes (*U.S. Nav. Med. Lab.* 1942 *b*), to over 45 minutes (Parker and MacNeal, 1944). The presence of saliva does not inhibit the destructive action of skin on dried virus (*U.S. Nav. Med. Lab.* 1943 *d*).

Virus Inactivating Power of Human Nasal Secretions

Burnet and his colleagues have shown that the serous secretion from the normal nasal passage has the property of inactivating influenza virus. The secretion is obtained by pushing a roll of gauze or cotton wool up the nostril. The material soon becomes soaked with secretion, and is wrung out with artery forceps to yield about 1 c.c. of clear fluid. Af ... rilized by heating to 56° C. for ½ hour, or by ... ane. The properties of the virus inactivating ... , Lush, and Jackson, 1939, Burnet and Lush, 1940 *b*, Francis, 1940 *a*)

1. It has been shown to inactivate various strains of virus A and the swine strain. It also inhibits CCA by virus (Soloviev and Parnes, 1946).

2. The inactivating effect proceeds slowly, and the degree of inactivation increases progressively as contact is maintained at 36° C; there is practically no effect at 0° C.

3. There is no evidence that virus inactivated by VIA can be reactivated by dilution

4. The effect of VIA is destroyed by boiling for 10 minutes, or 70–75° C. for 20 minutes

5 There is no correlation between lysozyme activity and VIA.

6. VIA is not a bile salt.

7. The capacity of the human nasal secretion to neutralize virus is not significantly influenced by deficiency of vitamins A or C, followed by "flooding" with the substances (Feller *et al.*, 1942).

8. Although there is no close parallel between VIA and the antibody titer of the serum, the majority of those with a low titer have no VIA, and *vice versa* most of
gests th
Soloviev
parallel

9 Francis and Brightman (1941) found that in 9/10 persons a natural infection with type A virus was followed by the development of a sharp increase in the capacity of their nasal secretion to inactivate virus Although not parallel, increases of neutralizing antibody in the serum were also observed.

10. As further confirmatory evidence that VIA is derived from serum antibody, it has been shown that persons given live virus in one dose subcutaneously develop a rise in serum antibodies and a concomitant enhancement of VIA; 2 inactivated vaccines produced similar effects (Francis *et al*, 1943).

Virus Inactivating Power of Animal Secretions

Immunization of rabbits has been shown to be accompanied by a rise in the virus neutralizing power of the nasal secretion, rising parallel with serum antibody (Soloviev and Parnes, 1946) These authors found no evidence that VIA was produced locally in the nasal cavities.

These observations lend still further weight to the belief that VIA is actually derived from serum antibody.

CHAPTER LXI

HEMAGGLUTINATION BY INFLUENZA VIRUS

PREPARATIONS containing any strain of influenza virus have the property of causing agglutination of chick and other varieties of red cell, as first shown independently by Hirst (1941)[1] and McClelland and Hare (1941).

Thus, Hirst found that if the red cells of an infected embryo gain access to allantoic fluid, agglutination takes place. When allantoic fluid is mixed in a test tube with washed adult chick cells, agglutination occurs, and the red cells sediment quickly to form a ragged granular pattern on the rounded bottom of the tube. Allantoic fluid, he found, may agglutinate even when diluted 1/512. This test is usually known as the chick red cell agglutination (or CCA) test, although other [illegible] that influenzal antibody specifically [illegible] and that antisera can be titrated by [illegible] red cell agglutination inhibition (or CCA-inhibition) test.

McClelland and Hare in their independent observations found that the red cells from a number of species are agglutinated by virus A and B, and that the virus is adsorbed to the red cell. The hemagglutinin can be destroyed by heat. They found a fair correlation between antibody titrations carried out by the CCA-inhibition and mouse tests.

These original observations have been abundantly confirmed, and extended, by Hirst, McClelland, Hare, their colleagues, and many independent observers. Burnet, in his study of the O-D phenomenon, and in other work, has made many interesting observations. The discovery of the Hirst-McClelland-Hare phenomenon of chick cell agglutination has led directly to a very considerable and rapid increase in our knowledge of the properties of influenza and other viruses, and has greatly simplified observations on immunity in natural and experimental infections.

SOURCES OF INFLUENZA HEMAGGLUTININ

Influenza hemagglutinin is most easily detected in allantoic or amniotic fluids from infected eggs. It can be found also in suspensions of infected chick embryo lung, as has been particularly shown by Burnet in his study of the O-D phase phenomenon. It can also be found in suspensions of mouse or ferret lung. Purified preparations of influenza virus, for example those obtained by centrifugation from allantoic fluid, can be standardized on the basis of their CCA potency per mgm. of nitrogen. Nasopharyngeal washings may agglutinate chick cells, and this can be inhibited by serum (Soloviev, Shubladze, and Meltzer, 1945).

SPECIES OF RED CELL AGGLUTINATED

The following are the reactions observed when preparations of influenza virus are mixed with the red cells of the most readily accessible species (McClelland and Hare, 1941, Burnet and colleagues, Twyble and Mason, 1944, Shubladze and Soloviev, 1945)

agglutination occurs—

chick (embryo, day-old, or adult), duck, pigeon, turkey, guinea-pig, human, dog, mouse, and ferret (weak),

[1] *References are appended at the conclusion of Ch. LXIII, p. 671 et seq.*

no agglutination occurs—

rabbit, rat, horse, sheep, fox, cat, and frog

Slight differences may occur using embryonic chick cells as distinct from adult cells, in experiments where cells are treated with virus and then exposed to the action of other viruses (Burnet *et al*, 1945, see below).

It will be remembered that Burnet claims that virus as originally isolated from the patient (O phase) does not readily agglutinate fowl cells, but agglutinates guinea-pig, human, or pigeon cells. Virus in its usual passage form (D phase) agglutinates chick and other red cells readily.

The most complete single series of observations is that of Clark and Nagler (1943) who obtained the results shown in Table 27 (to save repetition the reactions with 2 other viruses are also shown). They stress that this table gives a broad picture, and that some of the agglutinations are weak It will be observed that they find a difference in the agglutinating capacity of influenza virus strains, and also record as agglutinable some cells described as inagglutinable by others.

TABLE 27

REACTION OF RED CELLS TO INFLUENZA AND OTHER VIRUSES (CLARK AND NAGLER, 1943)

	B	*W S* (*A*)	*Melbourne* (*A*)	*Swine*	*Newcastle virus*	*Vaccinia virus*
Human	+	+	+	−	+	+
Sheep	+	−	+	−	+	±
Goat	+	−	+	−	+	±
Cow	+	−	−	−	+	+
Pig	+	−	−	−	−	+
Horse	+	−	−	±	−	−
Donkey	+	−	−	+	−	−
Cat	+	−	−	−	−	+
Ferret	+	+	+	−	−	+
Rabbit	+	+	+	+	+	+
Guinea-pig	+	+	+	−	+	+
Rat	+	+	+	−	+	+
Mouse	+	+	+	−	+	+
Ringtail opossum	+	−	−	−	+	−
Marsupial mouse	+	+	+	+	−	−
Hen	+	+	+	+	+	+
Duck	+	+	+	+	+	+
Sparrow	+			+	−	−
Frog	+	+	+	+	+	−
Tiger snake	+	+	+	+	+	−
Copperhead snake	+	+	+	+	+	+
Bluetongue lizard	+	+	+	+	+	−

PROPERTIES OF THE INFLUENZA HEMAGGLUTININ

Influence of Various Physical and Chemical Factors

Heat.

Hemagglutinin is destroyed by heat (60° C), but it is possible to expose virus to a lower degree of heat so that infectivity is lost before hemagglutinating capacity (Hirst, 1942 *c*, *d*) McKee and Hale (1946) found that concentrated virus heated to 57° C for 70 minutes was noninfective, but still possessed some agglutinating capacity. Lauffer and Carnelly (1945) have made a mathematical study of the process of inactivation of hemagglutinin by heat (61° C.). When the logarithms of residual agglutinating activities were plotted against times of heating, in the majority of cases one could not fit a straight line to the data. However, when the reciprocals of the square roots of the residual concentrations were plotted against the times, straight lines could generally be fitted. This relationship held for the

first 90–99 per cent. of the course of the reaction, but many cases of deviation were observed. After discussing various possible explanations of this behavior, the authors suggested that the virus particles (i.e., hemagglutinins) are inhomogeneous *with respect to the ease with which hemagglutinin is destroyed.* It has been found with other viruses that the rates of inactivation and denaturation vary inversely with concentration. The effect of initial virus concentration on specific reaction rates applied equally to the thermal destruction of influenza hemagglutinin.

It now appears that strains of virus may differ from one another in the stability of their CCA activity, and that the method of maintenance by passage is also of importance. Thus, Salk (1946 *a*) found that the Lee hemagglutinins withstood 61.5° C. for 90 minutes without a drop in CCA titer. The hemagglutinins of other strains tested were quickly destroyed at this temperature; strains differed one from another in this respect; lines of a single strain maintained in different hosts showed varying degrees of stability to heat, apparently due to an inherited property.

Francis (1947 *a*) found that 56° C. for 30 minutes did not alter the CCA titer of a number of B strains in allantoic fluid, although in 2 cases the titer was lowered. When the same strains maintained in mouse lung were heated similarly, their CCA activity was completely or markedly inactivated. Francis also found that this temperature did destroy the hemagglutinating capacity of several A strains, in distinction to his findings with B strains.

Storage.

On prolonged storage in the refrigerator, allantoic fluid may become noninfective, yet still cause agglutination of chick cells (Hirst, 1942 *c*). When stored at 37° C., infectivity is lost in a few days, but hemagglutinating activity remains for months (Pearson, 1944 *a*).

Stanley (1945 *b*) studied the effect of freezing formolized purified virus, and found that CCA activity was immediately lost. Drying from the frozen state resulted in loss of CCA activity.

Chemical and physical agents.

Preparations of formolized purified influenza virus as used in vaccines still cause hemagglutination, although they are of course noninfective. Salk (1946 *b*) found that formalin increased the heat stability of the hemagglutinins of a number of A and B strains, strains varying one from another in this respect. The hemagglutinins of 3 different lines of the Lee virus were, however, deleteriously affected in their heat stability by formalin. Excess formalin destroys CCA activity (Stanley, 1945 *b*). Virus rendered noninfective by mustard was found still to agglutinate red cells (Rose and Gellhorn, 1947).

When allantoic fluid is exposed to irradiation, infectivity is first destroyed, then the interfering power is lost, and finally the hemagglutinating capacity is lost on prolonged exposure (Henle and Henle, 1945 *d*). In more detailed experiments (1947) they reported that the HA titer of PR8 was unaltered for about 60 minutes of irradiation, loss of activity occurred rather rapidly at some time during the second hour. The HA of the Lee strain was more resistant, and survived for 2–4 hours, during the first 2 hours, the titer actually increased.

Hemagglutinin is not destroyed by bacterial contamination in egg fluids or organ suspensions.

The hemagglutinin of the Melbourne strain is resistant to the alpha toxin of *Cl welchii*, and to cobra venom, although vaccinia hemagglutinin is inactivated (Stone, 1946).

Miller (1944 *b*) has investigated the effect of pH in various buffer solutions on CCA activity and infectivity. He found that both properties are lost more rapidly on the acid than the alkaline side of the optimum pH. Under suitable conditions, infectivity decreases while the red cell agglutinating potency remains unaltered.

Both infectivity and CCA activity are removed together by filtration through a Seitz or Berkefeld filter (Twyble and Mason, 1944).

The Interaction between Virus and Red Cells

Influenza virus is adsorbed to red cells that it agglutinates, and can be later eluted therefrom, showing that the cell-virus union is reversible. Adsorption of virus to, and elution from, red cells has been used as a method of purifying virus (see p. 613), and of obtaining pure O phase virus (see p 595) The stages in this reaction are as follows

rbed to chick red cells from a virus-contain- the quicker and more complete is the ad- of virus adsorbed rapidly decreases when the cell concentration is below 1 per cent, with a 2 per cent. suspension, adsorption is virtually complete (Hare, Curl, and McClelland, 1946)

2. With PR8, hemagglutinins are adsorbed at 4° C. rapidly and almost completely. With increase in temperature, the adsorption is less complete, although taking place more rapidly. At 4° C. maximum adsorption occurs in 5 hours, at 27° C. in 5 minutes, and at 37° C. in 3–5 minutes (Hirst, 1942 *d*).

3. The activity of the portion of the cell responsible for the adsorption is not destroyed, although somewhat reduced, by heating to 100° C. for 5 minutes, adsorption occurs to the stroma of the cell (McClelland and Hare, 1941; Hirst, 1942 *d*) Apparently the nucleus of chick cells can also adsorb virus (Parodi, Lajmanovich, and Mittelman, 1944).

4 When fowl cells agglutinated by influenza virus, and left standing at room temperature for an hour or so, are shaken, they usually reagglutinate. A stable suspension of such virus-treated cells can be obtained by treating with specific homologous antiserum and washing in saline, presumably the serum combines preferentially with the virus and "frees" it from the red cell (Burnet, 1945, Burnet *et al*, 1945). Stabilization can also be obtained by allowing agglutination to occur at 37° C, and shaking the tube periodically. Eventually the cells become stable and linear scale (see below) cells without the use of

5. After a varying period, the adsorbed hemagglutinin elutes from the cell. The larger the amount of red cell suspension used to adsorb, the less the speed and extent of subsequent elution. The degree of elution at 4° C. is negligible in 18 hours, but increases as temperature rises. Almost all the adsorbed hemagglutinins are released in 4–6 hours at 23° C. with the Lee strain, and at 37° C. with PR8. The Lee strain is adsorbed and eluted more quickly than PR8 (Hirst, 1942 *d*) Within limits, variation in the volume of the eluent does not affect the yield of virus, although, of course, altering the titer of the fluid (Hare, Curl, and McClelland, 1946).

6 After red cells have fully adsorbed and eluted agglutinin, they cannot adsorb any further agglutinin, nor can such cells be agglutinated by fresh virus preparations of the same strain (Hirst, 1942 *d*) Presumably, the cell receptors in the erythrocyte responsible for adsorption of virus and consequent agglutination are altered in some way, even after elution, so that they can no longer adsorb fresh virus. Such cells may be fully agglutinable by other strains of virus.

By treating red cells with various strains, Burnet, McCrea, and Stone (1946) order, such that when a cell virus it is not agglutinated by following it. The order of viruses causing hemagglutination is as follows mumps, Newcastle virus, Melb, Mil A O, WS, Bel O, Ian O, Mil A D, Lee, Bel D, Ian D, swine, Hut B, and Mil B. The term "receptor gradient" of the red cell is used in this connection (see

also Burnet, 1945; Burnet *et al*, 1945). For example, red cells treated with Newcastle virus cannot be agglutinated by this agent, but are susceptible to agglutination by influenza strains.

7. Further evidence supporting the existence of these red cells receptors has been furnished by Friedewald, Miller, and Whatley (1947) whose work is described in detail on p. 638. They were able to obtain an extract from chick red cells that inhibits agglutination. When these extracts were made from red cells that had fully adsorbed and eluted virus (removal of receptors), no inhibition of agglutination could be demonstrated. It looked as if the inhibiting factor was identical with the receptor for virus hemagglutinin.

8. Virus-treated red cells become agglutinable by normal sera, very few human sera failing to agglutinate cells treated with swine virus to a titer of 160, guinea-pig and rabbit sera give a lower titer, the titer obtained with normal sera is roughly proportional to the position in the receptor gradient series of the virus used to prepare the cells (Burnet, McCrea, and Stone, 1946, Chu and Coombs, 1947). In general, the titer given by a normal serum is low for viruses early in the series and higher for those later. This is thought to be an example of the panagglutination of Thomsen.

9 The agglutinins can be adsorbed to, and eluted from successive batches of red cell, without any appreciable loss of CCA activity; the virus particles do not appear to be altered by their contact with the red cells (Hirst, 1942 *d*) Hirst has also studied the adsorption of influenza virus to living cells of the respiratory system (see p. 601).

10. Inactivated virus particles can be adsorbed and eluted (Hirst, 1942 *d*) Irradiated particles can also be adsorbed and eluted as long as the HA is not totally destroyed (Henle and Henle, 1945 *d*, 1947); cells treated with particles of this nature are refractory to agglutination by influenza and mumps viruses (1947)

11. The infective agent in virus suspensions is adsorbed simultaneously with the hemagglutinin, and 95 per cent of infectivity is removed in 15 minutes Thereafter the infective agent is gradually released from the cells, and after 4 hours the 50 per cent. mortality titer of the supernatant fluid is as high as at the beginning of the experiment (Hirst, 1942 *d*)

Effect of Bacterial Enzyme Products on Red Cell Receptors

The toxin of *Cl. welchii* Type A was found to render red cells (human) inagglutinable by influenza viruses. As contact continued, the red cell receptors were removed in roughly the order of the red cell gradient described above, i.e., after ½ hour of contact most strains were still able to agglutinate the red cells, after 3 hours only strains towards the right hand end of the scale were able to do so, and this effect was more marked after 6 hours (Burnet, McCrea, and Stone, 1946). These authors found that filtrates of the cholera *vibrio* had the same effect The susceptibility of the cells to agglutination by different viruses was progressively lost in almost the same sequence as resulted from virus action The only outstanding discrepancy was the undue retention of susceptibility to agglutination by mumps virus

This work rendered it highly probable that the action of influenza viruses on red cell receptors was also due to similar enzymes or "enzymatically active groupings" on the surface of the virus particle

Observations with Anti-Rh Sera

Chu and Coombes (1947) treated human cells with NDV and influenza strains, and then exposed the stabilized suspensions to antiserum containing the "incomplete" anti-D Rh antibody Only the Rh positive treated cells containing the D Rh antigen were agglutinated There was a gradation of activity, NDV being the strongest, and swine, Lee, and PR8 following These workers concluded that

viruses exert 3 sets of action on red cells (1) red cell receptors are removed, (2) panagglutinability is produced, (3) appropriate cells are rendered susceptible to agglutination by incomplete Rh antibody. As shown by Burnet, McCrea, and Stone (1946), and just mentioned, cholera vibrio filtrate has the same effect (see also Pickles, 1946).

The Relationship of CCA Activity to Infectivity and Other Properties

From what has been said, it will be evident that although the properties of infectivity and CCA potency of a preparation are usually closely related, they can be separated. For it is possible to destroy infectivity, for example, by heat, storage, irradiation, or formalin, and yet leave a preparation with practically unimpaired CCA activity.

Hirst (1942 *c*) showed that the CCA titer and mouse infectivity titer of a given strain are closely correlated in fresh, or carefully stored, preparations The CCA titers of increasing dilutions fall parallel to the mouse infectivity titers, when any given strain is titrated by the 2 methods If different strains are compared, the CCA titer by itself gives no indication of the likely infectivity. Hirst found that 4 strains in allantoic fluid had approximately the same CCA titer, but were of quite different virulence for mice.

There is no doubt that hemagglutination is brought about by the elementary bodies—the infective particles—of influenza virus, and not by any other fraction of the virus Centrifugation studies have shown that CCA activity is associated with the 600 S component and not with 30 S It will be remembered that the 600 S component is associated also with infectivity, and immunizing ability (see Ch. LVI) The interfering property of influenza virus is also associated with the elementary body (see p 621).

The application of heat to concentrated virus was found to cause a marked drop in hemagglutinating activity, but the ability of the inactivated virus to bind antibody was not lessened to the same extent (McKee and Hale, 1946)

When exposed to ultraviolet light, the HA is destroyed after infectivity, toxicity, and interfering capacity, but some complement fixing power remains when HA is destroyed (Henle and Henle, 1947).

CHICK CELL AGGLUTINATION INHIBITION

Antisera containing influenzal antibody inhibit chick cell agglutination (Hirst, 1941, McClelland and Hare, 1941) This inhibition is specific, and antiserum to virus A does not inhibit agglutination caused by virus B It appears that the inhibiting antibody is associated with the gamma component (Wyckoff and Rhian, 1945).

Burnet *et al* (1945) have studied the academic aspects of CCA inhibition They found that (*a*) the law of constant proportions holds over a wide range of concentrations, (*b*) a "time" effect can be clearly shown with highly diluted reagents, (*c*) equilibrium can be reached even in the presence of red cells They concluded that the reaction conforms to the general character of virus-antibody reactions Presumably virus and antibody combine, so that no free virus is available to agglutinate the red cells

The procedure adopted is to set up a series of test tubes containing a fixed quantity of known [illegible] ilutions of the serum under test. If the [illegible] tination will be inhibited in a varying [illegible] 50 per cent. agglutination is regarded as expressing the titer of the serum

The serum of ferrets, hamsters, and mice recovered from infection contains

CCA inhibiting antibodies, and these can also be stimulated by the intraperitoneal inoculation of allantoic fluid.

As influenza is so prevalent, the majority of "normal" human sera agglutinates chick cells to a low titer. On recovery from influenza, there is in most cases a marked production of antibody, so that the titer becomes several times higher than it was in the acute phase. The question of the correlation between the antibody titers found by various methods is discussed in Ch. LXI.

NONSPECIFIC AGGLUTINATION INHIBITION

Nonspecific inhibition of agglutination may occur under various circumstances, of which at least 5 have so far been identified. (1) the presence of a heat-labile factor in normal serum, (2) certain peculiarities of individual red cells, (3) the action of a possible heat-labile component in the virus particle normally preventing inhibition brought about by serum, (4) the action of polysaccharides, (5) the effect of red cell and tissue extracts

1. Normal human and animal sera may contain a factor, destroyed at 56° C in 30 minutes, which in low dilutions inhibits the agglutination of chick red cells by viruses A or B, thus mimicking the effect of specific antibody (Hirst, 1942 c, Beveridge and Lind, 1946).

Burnet and McCrea (1947) in experiments on O and D phases, found that normal ferret sera showed very high nonspecific inhibitory titers.

Certain of these normal sera inactivated O and D phase Bel. virus when inoculated in the amniotic or allantoic cavities respectively. Melbourne virus was not inactivated. Tests showed that both normal and immune inhibitors are probably present in the gamma globulin fraction The normal inhibitor of Bel (D phase) hemagglutination was precipitated at a slighter lower salt concentration than the inhibitor for strain Melbourne. The power to inhibit Melbourne hemagglutination was completely lost within 20 minutes on heating ferret serum to 62° C The inhibitory power against Bel. was much more resistant, and the inhibitory factor behaved very like immune body

This high inhibitory power of normal ferret serum against hemagglutination by the strain Bel. was not due to previous infection The experiments indicated a possible source of error in the interpretation of serological reactions with influenza viruses, and provided a new example of pseudo-immunological activity of a normal serum component. It was suggested that a proportion of serum globulin molecules appear to possess surface configurations reacting with "receptors" on red cells and virus particles.

McCrea (1947) showed that nonspecific inhibition of hemagglutination could be demonstrated in tests with strain Bel (D phase) and normal rabbit serum to titers of 100–320. He described a simple heat treatment which rapidly destroys the nonspecific hemagglutination inhibitor in rabbit serum, leaving the true immune inhibitor relatively unchanged Heating normal rabbit serum for 15–20 minutes at 62° C usually resulted in complete loss of nonspecific inhibitory properties The inhibitory activity fell off logarithmically with time The temperature coefficient was 2.2 for a 4° C. rise

The destruction rate of the specific inhibitory factor in immune serum was of a different order. At the time when the nonspecific inhibitory titer was completely destroyed, 75 per cent of the immune titer remained, and thereafter fell more slowly, 30–50 per cent of the original serum titer remained after heating at 62° C for 3 hours Fractionation experiments showed that both specific and nonspecific inhibitors were recoverable in the globulin fraction The inhibitory activity appeared associated with the gamma globulins.

It is of particular interest that a normal serum can behave in some respects like virus antiserum, and that this behavior is due to the normal serum gamma globulin It appears that the surface configuration of normal rabbit globulin resembles the

configuration of immune globulin (against the particular strain of virus used) sufficiently to give a pseudospecific interaction with the virus antigen. However, there are well-defined differences in heat stability. The thermal destruction of inhibitory activity in normal rabbit serum is accompanied by denaturation of the globulin, and complex formation between the denatured globulin and albumen.

2. Burnet and Stone (1946) found sharp differences between fowl cells from different birds in regard to the inhibitory titer of a heated serum containing no influenzal antibody. Only cells from 15 per cent of fowls were capable of demonstrating antibody titers below 10 with two B strains Later it was reported that for determination of antibody titers, the red cells should be obtained from fowls specially selected as yielding highly susceptible cells, a wide range of apparent inhibition titers was obtained with different cells using a human serum containing no antibody, but there was less variation in titer using a high titer serum (Ander-

olated B strains to be very susceptible

The same phenomenon was recorded

Francis has made important observations in this connection It was first shown that freshly isolated strains of B virus, in allantoic fluid, when used as antigen in CCA inhibition tests with normal serum gave very high antibody titers, approaching those observed with immune serum (Francis, Salk, and Brace, 1946). This inhibitory effect on agglutination was lost with most strains on passage by the allantoic route.

In later studies, Francis (1947 *a*) investigated the effect of heat (56° C. for 30 minutes) on the CCA activity of B strains, in general this had no effect on the CCA titer. However, when heated and unheated aliquots were used as antigens in CCA-inhibition tests with inactivated sera, a sharp disparity was observed.

The unheated virus antigen gave antibody titers for normal sera ranging from 0–64, and for immune sera from 128–1024 The heated antigen, with the same normal sera, gave apparent titers of 128–2048 The increases in titer in the immune sera detected by heated antigen were not in proportion

Francis postulated that influenza

stable and -labile components The

and reacts with specific antibody to

The heat-labile component reacts with, and neutralizes the effect of, a component of normal serum that tends to inhibit hemagglutination by virus In the presence of the intact complex in unheated virus, especially after a few allantoic transfers, the natural serum factor is countered by the labile component, and specific antibody can be measured If the labile component is absent, as in freshly isolated material, or after heating, the normal serum factor prevents agglutination and obscures the measurement of specific antibody

An enzyme produced by *V. cholerae*, that has been shown to parallel the action of virus on red cells in removing "receptors," also destroys Francis' normal serum

this inhibitor was

inhibitors mucus

he activity of the

ved by incubation

nhibitory effect of

polysaccharides.

4. Thus, the following have been shown to exert a considerable effect in inhibiting hemagglutination *in vitro:* flaxseed mucilage, citrus and apple pectins, blood group A substance, gum acacia, and gum myrrh (Green and Worley, 1947) Apple pectin has been found also to inhibit virus multiplication (see p. 628)

Inhibition by Red Cell and Tissue Extracts

5. A number of observations has been made on the inhibiting effect of red cell and tissue extrac· ... Thus, it has been found that extracts of red cells t ... virus may inhibit agglutination, presumably by ... intact red cell receptor; on prolonged contact of virus and inhibitor, the latter is progressively inactivated (Bovarnick and de Burgh, 1947; Green and Worley, 1947). Friedewald, Miller, and Whatley (1947) have carried out important studies in this respect.

(*a*) Inhibition of hemagglutination produced by the influenza (and mumps) virus was obtained in high titer with serum and tissue extracts of various human oragns (lung, liver, kidney, spleen), and organ titers were usually higher than serum titers. Heating to 75° C. for 30 minutes had no significant effect on the inhibiting substance in serum. In general, the titers of tissue extracts were reduced by heating to 65° C It seemed reasonable to conclude that at least part of the inhibition shown by serum and extracts of human tissues was due to a factor other than specific antibody.

(*b*) Comparable results were found with normal rabbit and guinea-pig tissues.

(*c*) Preparations of perfused organs were still inhibitory, but were usually reduced in activity two- to fourfold, suggesting that hemaglobin as well as tissue cells contained the inhibitory substance.

(*d*) Extracts of human, chicken, sheep, and rabbit red cells were prepared by mechanical disruption The supernatants were tested for capacity to inhibit agglutination of chick cells by influenza (and mumps) virus The findings clearly indicated a correlation between the capacity of the red cells to agglutinate with these viruses, and the inhibition titers produced by extracts of red cells. Thus, human ar ... extracts of these c ... poorly with the ... varied

(*e*) Extracts of human and chick cells did not neutralize the infectivity of influenza viruses, and did not fix complement.

(*f*) The substance in human cells responsible for inhibition was distinct from the A and B substances and the Rh factor

(*g*) The inhibitory agent in human and chicken cells was destroyed by heating at 65° C for 30 minutes

(*h*) Chicken red cells were treated with influenza virus at 37° C till they no longer agglutinated, having adsorbed and fully eluted virus Extracts of these cells did not cause detectable inhibition of agglutination It is thus seen that removal of receptors from red cells also removed the factor inhibiting red cell agglutination

(*i*) The simple explanation of nonspecific inhibition of virus hemagglutination is that the receptor substance released from red cells combines with virus and blocks the union of virus with red cells

There is no simple method available for differentiating nonspecific inhibition from that produced by specific antibody A rise in the serum inhibition titer, however, is undoubtedly due to specific antibody

(*j*) It appears that normal allantoic fluid has an inhibiting effect on virus hemagglutination (Svedmyr, 1946)

THE SIGNIFICANCE AND EXPLANATION OF VIRUS HEMAGGLUTINATION

A number of viruses causes hemagglutination, and these can be grouped as follows (Clark and Nagler, 1943, Burnet, 1945, Burnet *et al*, 1945, Burnet, McCrea, and Stone, 1946)

... The members of this group

hemagglutinins of influenza virus are identical with the virus particles, and can be eluted from agglutinated cells. Cells treated with mumps and Newcastle viruses are agglutinated by immune serum

It is suggested that the viruses in this group become attached to a single series of receptors which vary in their accessibility and the ease with which they can be removed by virus action, the two qualities are directly correlated. There is no suggestion of there being specific receptors for each virus (Burnet, 1945; Burnet *et al*, 1945).

Cl welchii Type A toxin, and filtrates of *V. cholerae*, both enzymes, change the surface character of human red cells so that they become progressively less sensitive to agglutination by viruses of this group The virus receptors are removed in almost the order of the receptor gradient (Burnet, McCrea, and Stone, 1946). Although there are certain differences between the activity of bacterial enzymes and the action of influenza viruses, it seems probable that agglutination by viruses of this group is brought about by enzyme-like agents. On the strength of finding

or by the enzyme of *V. cholerae* that has similar receptor destroying properties. It appears that influenza virus possesses an enzyme acting on glandular type mucin

2. *Variola-vaccinia-ectromelia* (see Ch. XXXI). These hemagglutinins can be readily separated from the EB's, and appear to be some product of the virus Cells which are susceptible to the action of viruses in this group are also agglutinated by suspensions of tissue lipoids This type of hemagglutinin cannot be eluted from the agglutinated cell

3 *Mouse pneumonia virus* (PVM, see Ch LXVIII).

4 *Fowl plague virus* (Lush, 1943)

TECHNICAL CONSIDERATIONS IN CCA AND CCA-INHIBITION TESTS

Before describing the technique to be followed in CCA and inhibition tests, to save repetition, a number of technical points must be considered

Red Cell Suspensions

Although many species of animal furnish red cells susceptible to the agglutinins of influenza virus, those of the fowl are usually used, and for most purposes are sufficiently reliable There is, however, a definite variation in susceptibility to agglutination of suspensions from different birds, and even of the same bird at different seasons (Stuart-Harris, 1943). When a large number of tests are being performed at once, the same batch of red cells should be used throughout To obviate this factor of varying agglutinability of red cells, Miller and Stanley (1944) recommend the parallel titration of an antigen of standard potency (see below).

with B strains Burnet om different fowls in luenza antibody, only titers below 10 with the particular B strains used.

The agglutinating titer of a virus preparation varies inversely with the concentration of red cells, i e, the stronger the cell suspension, the lower appears the titer of CCA activity According to Salk (1944), 0 5 c c. of a 0 25 per cent suspension

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1 *The mumps-Newcastle disease-influenza group* The members of this group

1944). On the basis of a number of experiments, Miller and Stanley (1944) concluded that 25° C. should be regarded as the standard temperature (see below).

Calculation of Titer

In the presence of virus, chick cells are agglutinated, and fall to the rounded bottom of the special agglutination tube. The red cells form a characteristic sediment at the foot of the tube, and the supernatant column of cell suspension becomes correspondingly clearer as the cells settle down. In Hirst's method, the titer of a virus preparation (or antibody titer of a serum) is read by comparing the density of the lower third of the tube with control standard tubes containing known percentages of cells in suspension, after a fixed period of 75 minutes at room temperature. Thus, if no agglutination occurs in a given tube, the density of its lower third will correspond to a standard representing 100 per cent. of cells in suspen-

of the cells

per cent. or

agglutinated,

that is to say, where 67–50 per cent of cells remain inagglutinated in suspension in the lower third, the reading can be made more accurately with a densitometer (Hirst and Pickels, 1942; Hirst, Rickard, and Whitman, 1942) or spectrophotometer (Schwartz *et al*, 1946). Davies (1947) describes a slide rule method for determining titers.

body titers are plotted on a geometric scale they give a fairly symmetrical probability curve.[1] If the same titers are plotted on an arithmetic scale, the arithmetic mean is not at the peak of the curve, and the value is greatly affected by a few sera of abnormal titer (Hirst et al., 1942) Schwartz et al. (1946) have devised an antibody index based on the arithmetic mean of pooled sera, and suggest that investigations on the population at large may afford a clew to the onset of an epidemic.

CONTROLS

In every test it is necessary to have one tube containing red cells and saline only, to make certain that the cells are not spontaneously agglutinable In CCA-inhibition tests an additional control is needed, to show that the virus preparation is causing agglutination in the absence of serum.

(Commission, 1946 *b*)

THE TECHNIQUE OF CCA AND CCA-INHIBITION TESTS

Hirst's Method

The following method can be thoroughly recommended (Hirst, 1942 *c*, Hirst and Pickels, 1942, Miller and Stanley, 1944)

These tests are performed in small thin "Wassermann" tubes measuring 7 cm by 0.8 cm These tubes must be of high quality and standard specification Each tube in a series must be exactly similar in bore and thickness of wall In tubes showing agglutination, the clumps of cells settle to the foot of the tube forming a "lacy" or granular sediment Correspondingly the supernatant fluid becomes clearer The test is read by assessing the

[1] The geometric mean between two titers a and $b = \sqrt{a \times b} = \frac{1}{2}$ (log a + log b), i.e., the figures are transformed into logs, added and divided by 2

is the optimum amount of red cells in CCA and CCA-inhibition tests. The majority of workers, however, use a 1.5 per cent. or 2 per cent. suspension.

In CCA-inhibition tests, the method should detect a small excess of unneutralized virus, and the lower the concentration of red cells, the smaller the amount of virus required to give a corresponding degree of agglutination, as shown by the pattern of the deposited cells, the concentration recommended by Salk is about the practical limit, as below this point the red cell deposit is too scanty to be read accurately (Burnet *et al.*, 1945).

Antigen

In CCA tests it is desired to estimate the potency of an unknown virus-containing fluid. Serial twofold dilutions are usually made, and an equal quantity of red cells added.

Lauffer and Carnelly [illegible] preparing serial twofold dilutions. [illegible] of virus-containing material is tr[illegible]ng 0.01 M phosphate buffer at pH 7, (i.e., a 1/100 dilution). Solutions containing relative concentrations varying by a twofold factor from 1/200–1/6400 are prepared each by single step dilutions of this 1/100 solution. Then 1 c.c. of a 1.5 per cent. cell suspension is added to 1 c.c. of each dilution, to give final relative concentrations of virus from 1/200–1/12,800. For samples of lesser activity, the first stage dilution is 0.1 c.c. to 2.4 c.c. solvent (1/25). Solutions with relative concentration between 1/50–1/800 are then prepared each by single step dilutions of this 1/25 dilution. By this method, errors are not accumulated as in the case of successive twofold dilutions.

In CCA-inhibition tests it is necessary first to obtain a preparation of virus and titra[illegible] use in such inhibition tests is usually furnished by t[illegible]ifuged allantoic fluid, but purified preparations are [illegible] 1944; Miller and Stanley, 1944). Fluid can be dried, e.g., by the lyophil process and later reconstituted with water (see Dudgeon *et al.*, 1946, Lépine, Sautter, and Reinié, 1946). Infected mouse lung can be used (Twyble and Mason, 1944). When performing a series of tests with unpurified allantoic fluid, the same egg fluid should be used throughout, or discrepancies will arise (see Stuart-Harris, 1943). Lower titers in the inhibition test are obtained using egg-adapted than recently isolated strains of virus as antigen, this variation is not so marked with high titer antisera (Stuart-Harris).

In inhibition tests, each tube contains virus, red cells and antiserum, arranged so that agglutination will occur except in those tubes where it is prevented by the presence of antiserum. Each tube must, therefore, contain at least one agglutinating dose of virus. The practice varies in this respect, and some use 2 or 4 such doses. It is essential for any one worker to be consistent in the number of agglutinating doses used.

Burnet *et al.* (1945) have studied this question. They point out that equilibrium should be reached in so short a time that it becomes immaterial in what order the reagents are mixed. The standard virus concentration should, therefore, be as high as is consistent with obtaining a clear end-point within the range of serum dilutions used. When a single agglutinating dose is used, a true equilibrium may not be reached.

Salk (1944) states that as long as constant proportions of virus and red cells are used, the CCA-inhibition titer of a serum remains constant. For example, if with a 0.125 per cent. cell suspension and 1 agglutinating dose of virus, the titer of the serum is 2560, it will remain the same if 0.25 per cent. red cells and 2 agglutinating doses are used.

Temperature

The agglutinated cells fall to the foot of the tubes more quickly at higher temperatures, but the rate of dissociation of the clumps is also more rapid (Salk,

1944). On the basis of a number of experiments, Miller and Stanley (1944) concluded that 25° C. should be regarded as the standard temperature (see below).

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In the presence of virus, chick cells are agglutinated, and fall to the rounded bottom of the special agglutination tube. The red cells form a characteristic sediment at the foot of the tube, and the supernatant column of cell suspension becomes correspondingly clearer as the cells settle down. In Hirst's method, the titer of a virus preparation (or antibody titer of a serum) is read by comparing the density of the lower third of the tube with control standard tubes containing known percentages of cells in suspension, after a fixed period of 75 minutes at room temperature. Thus, *if no agglutination occurs in a given tube, the density of its lower* third will correspond to a standard representing 100 per cent of cells in suspension. On the other hand, if maximum agglutination occurs, the density of the cells *remaining in suspension in the lower third of the tube will be only 25 per cent.* or less. The end-point is that dilution where 33–50 per cent. of cells are agglutinated, that is to say, where 67–50 per cent. of cells remain inagglutinated in suspension in the lower third, the reading can be made more accurately with a densitometer (Hirst and Pickels, 1942; Hirst, Rickard, and Whitman, 1942) or spectrophotometer (Schwartz *et al.*, 1946). Davies (1947) describes a slide rule method for determining titers.

In other methods, the titer is calculated by observing the pattern of the clumped *cells at the foot of the tube after complete settling, this is facilitated by the use of* a mirror under the rack (Lépine, Sautter, and Reinié, 1946).

Titers can be expressed in terms of their common logarithms if desired. If antibody titers are plotted on a geometric scale they give a fairly symmetrical probability curve[1] If the same titers are plotted on an arithmetic scale, the arithmetic mean is not at the peak of the curve, and the value is greatly affected by a few sera of abnormal titer (Hirst *et al*, 1942) Schwartz *et al.* (1946) have devised an antibody index based on the arithmetic mean of pooled sera, and suggest that investigations on the population at large may afford a clew to the onset of an epidemic.

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albumin sac, the contents of which enter the amniotic cavity at about the 13th day (Commission, 1946 *b*).

THE TECHNIQUE OF CCA AND CCA-INHIBITION TESTS

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Correspondingly the supernatant fluid becomes clearer The test is read by assessing the

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Antigen

In CCA tests it is desired to estimate the potency of an unknown virus-containing fluid. Serial twofold dilutions are usually made, and an equal quantity of red cells added.

Lauffer and Carnelly (1945), however, recommend the following method of preparing serial twofold dilutions. For samples of high agglutinating activity, 0.1 c.c. of virus-containing material is transferred to 9.9 c.c. of physiological saline containing 0.01 M phosphate buffer at pH 7, (i.e., a 1/100 dilution). Solutions containing relative concentrations varying by a twofold factor from 1/200-1/6400 are prepared each by single step dilutions of this 1/100 solution. Then 1 c.c. of a 1.5 per cent. cell suspension is added to 1 c.c. of each dilution, to give final relative concentrations of virus from 1/200-1/12,800. For samples of lesser activity, the first stage dilution is 0.1 c.c. to 2.4 c.c. solvent (1/25). Solutions with relative concentration between 1/50-1/800 are then prepared each by single step dilutions of this 1/25 dilution. By this method, errors are not accumulated as in the case of successive twofold dilutions.

In CCA-inhibition tests it is necessary first to obtain a preparation of virus and titrate it by a CCA test. Antigen for use in such inhibition tests is usually furnished by the supernatant of lightly centrifuged allantoic fluid, but purified preparations are probably more suitable (Salk, 1944; Miller and Stanley, 1944). Fluid can be dried, e.g., by the lyophil process and later reconstituted with water (see Dudgeon *et al.*, 1946; Lépine, Sautter, and Reinié, 1946). Infected mouse lung can be used (Twyble and Mason, 1944). When performing a series of tests with unpurified allantoic fluid, the same egg fluid should be used throughout, or discrepancies will arise (see Stuart-Harris, 1943). Lower titers in the inhibition test are obtained using egg-adapted than recently isolated strains of virus as antigen, this variation is not so marked with high titer antisera (Stuart-Harris).

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Temperature

The agglutinated cells fall to the foot of the tubes more quickly at higher temperatures, but the rate of dissociation of the clumps is also more rapid (Salk,

3 Add 1 c.c. diluted antigen to tube 1 and to tube 2 Mix the contents of tube 2 thoroughly, and transfer 1 c c to the third tube Mix and repeat the process, discarding

an be
final

Tube	1	2	3	4	5	6	7
Dilution							
1 in	32	64	128	256	512	1024	2048

5. Incubate at room temperature for 75 minutes in a secluded part of the room. The tubes must not be disturbed at all during this period, and must be absolutely vertical

6. Assess the degree of agglutination in each tube by reference to the set of standards The titer of the antigen is the last tube to show 50 per cent. (++) agglutination This point may fall midway between two tubes

Method of antibody titration.

Before sera can be titrated, it is necessary first to set up an antigen titration as described above

1 All sera should be inactivated at 55° C. for 20–30 minutes

2 Dilute the serum under test ⅛ (0.25 c c. + 1 75) unless the titer is known to be high, when a different dilution may be used When using normal serum, dilute ½.

3 Rack out 8 tubes and 1 control Add saline 0 5 c c to all tubes except no 1 and to the control. Add 0 5 c c diluted serum to tube 1 and to tube 2 Carry 0 5 c c. from tube 2 along the row and discard from tube 8, thus preparing doubling dilutions of serum from ⅛ upwards.

4 Divide the titer obtained in the previous antigen titration by 16, and make up a corresponding dilution in saline. For example, if the antigen titered 1/256 the dilution prepared would be 1/256 divided by 16, equalling 1/16 (0 5 c c + 7 5 c c.).

5 Add 0 5 c c. of this diluted antigen to all tubes and to the control

6 Add 1 c c. of 1 5 per cent. cell suspension to all tubes and control The final dilution of antigen in all tubes is now 4 times that giving ++ agglutination in the original titration, i e , 4 agglutinating units are used.

The final dilutions of serum are as follows

Tube	1	2	3	4	5	6	7	8
Dilution								
1 in	32	64	128	256	512	1024	2048	4096

7 The tubes on the left hand side will probably show no agglutination, but somewhere in the row agglutination will begin. The tube showing 50 per cent (++) agglutination gives the titer of the serum. Frequently the transition is very abrupt, and no tube may actually show ++ agglutination The end-point is then taken as halfway between a tube of density higher than 67 per cent and an adjacent tube of density lower than 50 per cent

8 The control, containing 0 5 c c saline, 0 5 c c antigen and 1 c c cells should show marked agglutination, the density of the lower third being probably 25 per cent or lower

Other Methods

1. Method of Burnet (see Burnet *et al*, 1945).

Burnet uses round-bottomed test tubes of 1 cm internal diameter. Dilutions of serum or virus are made in twofold steps, 5 agglutinating doses of virus are used The final test consists of 0 25 c c virus dilution, 0 25 c c serum or saline, and 0 25 c c of 2 per cent red cells The tubes are shaken and left at room temperature, or sometimes at 4° C, till the red cells have settled The end-point of virus activity, or the serum titer, as the case may be, is read as that degree of partial agglutination arbitrarily regarded as being +. This degree of agglutination is characterized by a central button of inagglutinated cells, surrounded b

Burnet anc

(see below)

giving titers l

suspension The virus was diluted to give one standard agglutinating dose with 2 per cent.

degree of "clearing" of the lower third of the tube as compared with standards. In strongly positive tubes, under 25 per cent. of cells remain in suspension. In weaker tubes 50 per cent or more of cells remain in suspension. In negative tubes there is little clearing of the lower third of the tube and only a small compact disk of cells that have settled to the foot of the tube. These tubes should match the 100 per cent. standard.

Fowl cells.

A fowl (aged 6–18 months) should be bled into citrate-saline. The blood is filtered through muslin and then washed 3 times in saline. Finally the washed cells are "packed" These cells can be used for several days if stored in the refrigerator For rapid spot-testing of allantoic fluid, a 10 per cent. suspension in normal saline is used, but for accurate titrations a 1.5 per cent suspension. For accurate work, a portion of the 1.5 per cent. suspension may be centrifuged in a hematocrit tube to check the actual strength, which can then be corrected if necessary.

Standards and reading results.

In the test proper, one volume of red cell suspension is diluted with one volume of [illegible] 100 per cent of this [illegible] the lower third will [illegible] o per cent. standard [illegible] cent. of cells remain- [illegible]

The standards are prepared as follows

Standard Represents Percentage of Cells in Suspension	*Add Saline, c c*	*Add 1 5 Per Cent Cell Suspension, c c.*
100	1	1
87	1 13	0 87
75	1 25	0 75
67	1 33	0 67
50	1 50	0 50
37	1 63	0 37
25	1.75	0 25

The degree of agglutination is assessed by comparing the lower third of each tube with the above standards. The tubes should be read against an illuminated white ground, and must be handled very carefully so as not to disturb the sediment The standards are, of course, well shaken immediately before use, as any sedimentation would affect their correct strength The results are read as follows

Density of Lower Third of Tube, Percentage of Cells in Suspension	*Degree of Positivity*
Between 100 and 87	−
Between 87 and 75	±
Between 75 and 67	+
Between 67 and 50	++ (the end-point)
Between 50 and 37	+++
Below 37	++++

The end-point is arbitrarily taken as the last dilution in which ++ agglutination occurs No one tube may show ++ agglutination, the point falling between 2 tubes The titer is then taken as halfway between these points.

Method of antigen titration.

Various virus-containing suspensions can be titrated for their CCA potency by preparing falling dilutions, and adding a standard volume of cells Thus amniotic fluid, allantoic fluid, organ suspension, and purified virus preparations can all be tested by the following method, the supernatant being used after light centrifugation

1 Dilute antigen 1/16, if approximate titer is unknown (0 25 c c antigen plus 3 75 c c saline). If the strength is known to be high, this dilution could be increased to 1/32 or 1/64.

2 Rack out 7 tubes for the test, and 1 for the control Add saline 1 c c to all tubes except tube 1, and to the control.

that when the logarithm of activity was plotted against the logarithm of relative concentration, the data fitted a straight line with a slope of unity, as they should if the titration was reliable over a range of concentrations

5. Method of the U.S Army Medical Department.

This method has been recommended so that the results of inhibition tests obtained by various centers may be comparable (*Bulletin*, U.S Army Med Dept., 1946, Whitman, 1947). The total bulk of fluid is 0 75 c c representing equal portions of allantoic fluid (4 agglutinating units), serum dilutions, and 1 per cent human O cells. Recordings are made after 45 and 120 minutes

cells (equivalent to 4–5 doses with 0.5 per cent. cells). The end-point was a standard degree of partial agglutination read after settling of the cells, reshaking, and a final settling, at room temperature throughout.

Guinea-pig cells are said to give results that can be read more easily. It is necessary first to absorb the serum (1/10) with 10 per cent of packed cells, to remove agglutinins for guinea-pig cells, present in most normal human sera (Beveridge and Lind, 1946).

2. Method of American Naval Laboratory No. 1.

For inhibition tests these workers recommend the use of a constant 1/100 dilution of unknown serum, a constant amount of human group O cells, and falling dilutions of known virus (*U.S. Nav. Med. Lab.*, 1943 *b*, Van Gelder, Greenspan, and Dufresne, 1947). The antibody titer is expressed by comparing the amount of virus which causes an arbitrarily selected degree of agglutination in the presence of normal and immune serum.

In CCA tests these workers mix 0.5 c.c. of each twofold dilution of virus with 0.5 c.c. 1 per cent. normal rabbit serum in saline, and then add 0.1 c.c. of 0.75 per cent. of washed human O cells, or chick cells. The serum prevents the formation of a film of cells on the bottom of the tubes, which interferes with the reading of negatives and controls. 0.1 per cent gelatin in saline can be substituted for serum (*U.S. Nav. Med. Lab.* No. 1, 1944 *a*).

3. Salk's (1944) method.

Salk prepares serial twofold dilutions of virus in saline, and adds 0.5 c.c. to tubes as recommended by Hirst. To each tube, 0.5 c.c. of 0.25 per cent. washed chick cells is added. The tubes are well shaken and set aside till the cells are completely settled (½–2 hours). In negative tubes, there is a central sharp disk of cells. In tubes showing maximum (+) agglutination there is a uniform film of clumps of cells covering the entire bottom. Intermediate degrees are scored as ±. The titer of the virus is given as the reciprocal of the highest final dilution giving maximum (+) agglutination.

In CCA-inhibition tests, Salk recommends the use of an antigen partially purified by adsorption and elution. He prepares serum dilutions in 0.25 per cent. red cell suspension, from 1/32–1/1024 in samples from acute cases, and 1/32–1/16,000 in convalescents. To 0.5 c.c. of each serum dilution he adds 0.5 c.c. of virus suspension of twice the highest concentration needed to give maximal agglutination. The test is read by studying the cell deposit at the foot of the tubes. The titer is the reciprocal of the final dilution of serum in which there is complete inhibition of agglutination.

By this method, normal sera usually show a titer below 128, and convalescent sera from 256–2048.

4. Miller and Stanley's (1944) standardized technique.

Miller and Stanley (1944) investigated the variable factors in the CCA test, with a view to introducing a standardized test to serve as an index of chemical purity. They found that the variation in the results of the agglutination tests could be attributed to varying behavior of different batches of red cell, and to temperature. They suggested that 25° C. should be regarded as the standard temperature for performing CCA tests, and furnished a formula to enable the titer at this standard temperature to be derived from the titer at room temperature.

They recommended that in any series of titrations of unknown virus samples a standard purified preparation should be included. They found that purified virus kept at 4° C. remained stable for several months, and gave results in repeated tests which only varied on account of the behavior of the red cells. The average titer of the B9 preparation of virus was 26,000 units per mgm. of protein nitrogen, converted to the standard temperature of 25° C. The standard procedure recommended involves:

Parallel titration of a standardized preparation of virus along with the unknown virus samples.

The CCA activity of the unknowns is then obtained by multiplying the value of their observed titers by the ratio of the theoretical titer of the standard to the observed titer of the standard. The effect of variations due to red cells and temperature are thus cut out.

To obtain the highest degree of accuracy, duplicate titrations should be performed. They found that the chances were 19/20 that differences of 8.4 per cent. between the mean end-points were significant. Lauffer and Carnelly (1945) confirmed this, and found

gen-antibody reaction, using egg virus and immune ferret serum, with the chorio-allantois as the indicator of neutralization (1936 *d*, Burnet, Keogh, and Lush, 1937). He found that there are 2 stages in the interaction between virus and serum. First, equilibrium rapidly becomes established, in the early stages this reaction is reversible. Second, there occurs a slower phase of irreversible inactivation

(*b*) *In the allantoic cavity.* Virus-serum mixtures can be titrated by inoculation in the allantoic cavity. The presence of chick cell agglutination after incubation is taken as denoting failure of neutralization. If 6 embryos are used per dilution, a 50 per cent. end-point can be calculated (Hirst, 1942 *a*, Burnet, 1943 *b*; Bull and Burnet, 1943, Beveridge and Williams, 1944). Examining sera from cases of influenza, Burnet and Beveridge (1943) found that this method recorded a much greater rise in antibody in convalescence than was shown by the CCA-inhibition method.

Burnet and Stone (1946) in work with virus B, used the following technique (see also Beveridge and Burnet, 1946) Mixtures of serum and virus are held in contact for one hour at 4° C. and inoculated in 0 05 c c quantities in the allantoic cavity of 11-day embryos After 3 days incubation at 35° C., a small amount of allantoic fluid from each egg is tested for hemagglutination. With sera containing little antibody, tests are made with mixtures of undiluted serum plus serial tenfold virus dilutions With active sera, virus diluted 1 100 is mixed with equal volume of serum diluted 1-, 3-, 10-, 30-, and 100-fold. Six eggs are used for each mixture, and the 50 per cent end-point is calculated by Reed and Muench's method. The activity of a serum is expressed as the dilution which mixed with 1:100 virus will produce 50 per cent of infections in 3 days. Allowing for the twofold dilutions of virus in all mixtures, the standard virus (B) used in this work had a titer of $10^{6.3}$ With sera which undiluted fail to neutralize virus diluted 1 100, the titer is expressed according to a convention based on the fact that the neutralization curve, when logarithmic concentrations of virus and antibody are used as ordinates and abscissae respectively, is a straight line with a slope of 3 6 1. A serum, therefore, which gives a 50 per cent end-point with virus 1/105, will be ½ the strength of one giving an end-point with the standard 1/100 dilution

When the titer of the virus differed from the value $10^{6.3}$ taken as standard, the serum titers were adjusted according to the formula: $\log S^1 = \log S + \frac{\log T - 6.3}{3.6}$, where S^1 is the corrected serum titer, S the crude serum titer, and T the virus titer. From the work of Knight (1944 *b*, see p 619), it appears that 10 embryos should be used to justify the use of this correction factor.

Virus neutralization by the CCA method.

This technique is fully discussed in Ch LXI. For the sake of completeness, it may here be mentioned that the influenza virus-antibody reaction, as studied by CCA-inhibition, conforms to the general character of such reactions studied *in vivo* or on the chorio-allantois (Burnet *et al*, 1945).

Correlation between neutralizing titers determined by different methods.

There is, of course, at any one examination, no numerical correspondence between the antibody titers found by the CCA-inhibition test, by the mouse infectivity test, and by inoculation in the allantoic cavity. This is not to be construed as meaning that different antibodies are concerned with the 3 reactions. It signifies only that the tissues or cells tested vary widely from one another in their demonstrable susceptibility to influenza virus For instance, Burnet (1943 *a*) tested virus-serum mixtures by the CCA-inhibition test, by allantoic inoculation, and by chorio-allantoic pock counting The CCA-inhibition test showed the greatest evidence of

CHAPTER LXII

IMMUNITY IN INFLUENZA—ACADEMIC AND EXPERIMENTAL ASPECTS

ANTIGEN-ANTIBODY REACTIONS

Virus Neutralization

INFLUENZA antibody neutralizes the infectivity of virus, and this can be demonstrated by different methods *in vivo; in addition*, the CCA-inhibition technique can be employed This has already been fully described (see above).

Virus neutralization tests in animals.

In this type of test, the serum is diluted serially and mixed with a fixed quantity of virus. Alternatively, the serum can be kept constant, and the virus diluted serially. After contact *in vitro* (e g, 1 hour at 37° C and 1 hour at 4° C.), the mixture is inoculated intranasally in mice. Autopsies are performed as animals die, and survivors are killed after 10 days. The lungs are examined for the presence of lesions due to the virus The 50 per cent end-point can be calculated from specific deaths, or from the presence of influenzal lesions in the lungs (see, for example, Horsfall, 1939).[1]

Burnet and Clark (1942) recommend the use of dilutions of immune sera with stock virus obtained from a mouse lung suspension They take as end-point the production of consolidation between ⅙ and ½ of the visible lung surface (thorax opened from the back).

The titration experiments of Horsfall showed that a linear relationship exists between the logarithm of the quantity of virus (PR8) neutralized, and the logarithm of the quantity of animal serum capable of achieving this effect A fivefold dilution of serum was found to result in a tenfold decrease in quantity of virus neutralized. Horsfall and Lennette (1941) found that a linear relationship exists also between the quantity of human serum and the amount of virus A neutralized It is possible, therefore, to determine the maximum amount of virus a given serum can neutralize. This neutralizing capacity is a fixed value, and is independent of the amount of virus used in the neutralization test.

McKee and Hale (1946) have shown that mixtures "apparently neutral" by their failure to produce lesions in mice may not really be neutral, for passage of the lungs may "unmask" active virus They distinguish between "apparently neutral" mixture and "absolutely neutral" mixtures, where no virus is unmasked by serial passage These workers also showed that alternate freezing and thawing did not reduce the protective power of antiserum in mice

Antisera will also neutralize the toxic effects of influenza virus, and this effect is type- and to a lesser extent strain-specific (Henle and Henle, 1946 *a, b*)

Virus neutralization tests in eggs.

(*a*) *On the chorio-allantois* Certain influenza strains produce characteristic pocks on the chorio-allantois, and this property is inhibited by antiserum Mixtures of serum and known virus preparation are inoculated on the chorio-allantois of 12-day embryos After a suitable period of incubation, the eggs are opened, the membranes removed and pocks counted (see, e g., Burnet and Foley, 1940, Burnet and Lush, 1940 *c*) Burnet has carried out important academic studies on the anti-

[1] References are appended at the conclusion of Ch LXIII, p 671 et seq

neutral mixtures, even after prolonged interaction, and irrespective of the amount of antibody present These authors suggest, therefore, that there is no irreversible destruction of virus by antibody The fact that the amount of virus recovered from virus-antiserum mixtures is less than that recovered from virus-normal serum mixtures can be explained in two ways Probably the methods are not perfect, and better technique could detect a greater quantity of virus Or perhaps virus particles are agglutinated by the serum, and carried down on precipitable nonviral tissue.

There seems little doubt, therefore, that antibody does not completely inactivate all the virus particles in "neutral" mixtures The question in dispute is whether a proportion of virus particles is eventually completely inactivated, or whether improved technique would result in all the original infectivity being recovered from such mixtures.

Complement Fixation

It has been known for many years that complement fixation occurs between antiserum and influenza virus; and it was early recognized that the complement fixing and infective powers are partly independent (Smith, 1936, Hoyle and Fairbrother, 1937 *a*, *c*, Lush and Burnet, 1937, Fairbrother and Hoyle, 1937 *a*, Francis *et al*, 1937 *a*, *b*, Tulloch, 1939).

Various materials can be used as antigens in complement fixation tests, for example, tissue (especially lung) extracts, or tissue cultures Of recent years, infected allantoic fluid has been much used (Nigg, Crowley, and Wilson, 1940, 1941; Nigg, Wilson, and Crowley, 1941) These antigens owe their complement fixing properties to 3 components that have been separated by centrifugation (see p. 587) First, the elementary bodies themselves (the 600 S component) can fix complement Second, the soluble substance or 30 S component, remaining in suspension when the EB's have been deposited, also fixes complement. Finally, another component known as < 30S, which remains suspended when 30 S has been thrown down, also fixes complement Of these [illegible]

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hancing factor has been found in many human and animal sera Complement should, therefore, be titrated under the conditions of the test. Hoyle (1945) analyzed the CF reaction in influenza by means of the "chessboard" type of experiment, a series of antigen dilutions being tested against a series of serum dilutions The results were plotted graphically, the curve being obtained by drawing a line through all mixtures giving 50 per cent hemolysis. The maximal serum and antigen titers and the optimal antigen titers could thus be estimated When the ratio of maximal to optimal antigen titer was known, it was possible to derive the optimal antigen titer from the maximal titer, and this could be found by a simple titration of antigen with excess serum (see also the studies of Rice, 1947).

In this work, Hoyle confirmed earlier observations that the reaction was complex, and involved the elementary body and the soluble antigen.

The optimal titration technique has also been recommended by Wiener, Henle and Henle (1946), and obviates the zone phenomenon

The pH mobility of soluble substance was found to be definitely smaller than that of serum albumin, and was close to that of alpha globulin, with an iso-electric point close to pH 5 (Bourdillon and Lennette, 1940).

The development of soluble substance has been studied in saline suspensions of ground lungs of mice inoculated intranasally with PR8 (Eaton and Nicewonger, 1940) The antigen appeared in the lung even after one day, although there were no visible lesions The maximal amount of antigen was probably formed during the period of rapid multiplication of the virus, before the appearance of consolidation The antigen disappeared from the lungs after 7–8 days

neutralization, and the allantoic test the least evidence. This implies that the chick red cell is considerably less susceptible to the effect of a given quantity of virus than is the allantoic cavity, which has numerous cells easily invaded by virus.

The effect of antiserum on the infectivity of virus.

It has been described how the neutralizing effect of influenzal antiserum on virus can be demonstrated by a number of techniques. Although the virus in mixtures shown to be neutral by such tests exerts no apparent pathogenic effect, the question arises as to whether or not it is actually destroyed by the antiserum. A number of observations have been made on this point, from which it appears that the action of antiserum on virus is partly reversible in the early stages, but becomes irreversible later, with apparent destruction of at any rate a portion of the virus.

Simple dilution can render infective certain mixtures of serum and virus which are noninfective for mice in more concentrated form; however, only about 10 per cent. of the original infectivity can be restored by such a procedure (Taylor, 1941 *a*; McKee and Hale, 1946). Similar observations have been made by Burnet in eggs (see below).

Papain digestion also reactivates neutral mixtures (McKee and Hale, 1946).

Neutral mixtures do not usually contain enough free antigenic material to stimulate active immunity in mice (Lyons *et al.*, 1944). Further, the treatment of a formolized preparation of virus A with homologous antiserum largely suppresses the capacity of the virus to stimulate the production of antibody or resistance to infection (Beveridge, Stone, and Lind, 1944).

Working with tissue cultures, Magill and Francis (1937) found that chick embryo cells will support the growth of virus when exposed to the action of a potent antiserum, and then washed. Virus is able to survive if it is added to the cells before a relatively weak antiserum. However, if virus and weak serum are mixed before adding to the culture, little growth takes place. With a powerful serum no growth occurs, irrespective of whether the virus or serum is first added to the cells.

The problem has also been studied in eggs. Using strains adapted to amniotic passage, Burnet (1941 *a*) found that immune serum exerted very little neutralizing effect; the embryo might survive for a longer time, but showed the characteristic effects of infection with virus. On the other hand, when strains not specially adapted to amniotic passage were used, neutralization could be demonstrated.

In similar studies, Burnet (1943 *a*) tested the neutralization of virus by allantoic inoculation. The neutralizing power of serum was found to decrease rapidly on dilution, and with suitable mixtures it was easy to demonstrate reactivation by dilution. Even so, there was evidence that some of the virus was rendered inactive. Virus strains well adapted to growth in the allantoic cavity are more difficult to inactivate by serum than strains less adapted to this route.

Mixtures found to be "absolutely neutral" by serial mouse passage could be reactivated by the addition of concentrated heat-inactivated homologous virus, about 10 per cent. of the original virus was reactivated (McKee and Hale, 1946).

Smorodintseff and Shiskina (1946) in an extensive study have compared various methods of recovering virus from neutral mixtures. (1) Dilution was not sensitive enough to "unmask" virus from overneutralized mixtures, although it could be recovered when relatively little antibody was present. (2) Using electrophoresis, virus could be recovered only from mixtures containing a small excess of antibody. (3) Virus could readily be recovered, even from overneutralized mixtures, by adsorption on kaolin or carbon, and inoculation in the form of a suspension in the nares of mice. (4) The most efficient methods of recovering virus were (*a*) filtration through Elford filters (APD 125–175 mμ), grinding the filter pad, and injecting mice, (*b*) centrifugation at 13,000 r.p.m., and washing the deposit prior to animal inoculation.

They showed that a considerable quantity of virus could be recovered from

Forssman Antigen

It should be remembered that Forssman antigen may be present in virus preparations purified by centrifugation of allantoic fluid (Henle *et al*, 1944) Further, rabbit sera prepared by inoculation of virus purified by centrifugation from allantoic fluid may contain Forssman antibody (Knight, 1944 *c*).

ACTIVE IMMUNITY IN EXPERIMENTAL ANIMALS

Active Immunity Induced by Live Virus

Ferrets and mice infected with influenza virus by nasal inoculation prove, on recovery, to be resistant to reinfection for several weeks (see Smith, Andrewes, and Laidlaw, 1933, 1935, Francis and Magill, 1935 *b*, Burnet, 1938, Francis, 1939, Oakley and Warrack, 1940; Eaton and Beck, 1940, Burnet and Clark, 1942, Sugg and Magill, 1946, 1947). When immunity begins to wane, it can be reinforced by vaccination.

Active immunity can be stimulated by the inoculation of live virus by routes other than the intranasal. Mice can be immunized by subcutaneous injection of live virus (Smith, Andrewes, and Laidlaw, 1935, Rickard and Francis, 1938, Andrewes and Smith, 1939). As little as 2 injections of 0·0025 c.c. of neat infected allantoic fluid are sufficient (Burnet, 1941 *c*). Mice can also be immunized by the intraperitoneal route (Burnet, 1938, Rickard and Francis, 1938, Andrewes and Smith, 1939, Francis, 1939, Eaton, 1940 *a*, Eaton and Pearson, 1940, Oakley and Warrack, 1940). Thus, Rickard and Francis injected large doses, and found that mice were immune to nasal inoculation 24–48 hours later. Francis found that a direct relationship existed between the concentration of virus given intraperitoneally and the degree of immunity to nasal inoculation.

Ferrets can also be immunized by subcutaneous or intraperitoneal injections (Smith, Andrewes, and Laidlaw, 1935, Andrewes and Smith, 1939, Francis, 1939). Francis gave subcutaneous injections of live virus, and found that approximately 100 or more intranasal infective doses were needed to give a partial immunity, whereas one infective intranasal dose produced a firm and immediate immunity.

Monkeys have been rendered resistant to a symptomatic infection by the intratracheal inoculation of live virus (Burnet, 1941 *b*).

Less live virus is needed to immunize mice against the homologous strain than against heterologous strains (Smith and Andrewes, 1938, Burnet, 1938). For example, Eaton and Pearson (1940) working with 3 A strains injected intraperi-
[illegible]

strains of PR8, Phila, and a tissue culture strain of swine virus. This dosage gave a solid resistance to infection with the heterologous strains However, when minimal amounts were given, the immunity might be effective only against closely related strains Sugg and Magill (1946) found that ferrets infected intranasally resisted a challenge inoculation of the homologous virus 6 weeks later, but were not resistant to a closely related A strain However, if the animals had been infected twice with the homologous strain they resisted a 3rd inoculation of the related strain. They also showed that ferrets recovered from infection with virus A or B resisted the homologous virus as early as the 8th day of convalescence, but were susceptible to the heterologous strain (1947).

Immunity can also be induced by living virus of lowered virulence. For in-

The peak of complement fixing power of allantoic fluid occurs at 36 hours after inoculation (Henle and Chambers, 1941). Infected allantoic fluid contains both the 30 S component, and the 600 S or elementary body component, but contains much less 30 S than mouse lung extracts (Friedewald, 1943, Friedewald and Pickels, 1944).

Complement fixing activity is completely destroyed at 63° C. and partly at 56° C. (Lennette and Horsfall, 1940). The activity of irradiated allantoic fluids as antigens in the CFT was found to be more stable than their HA capacity, the antigen titer being unaltered after 120 minutes irradiation. Thereafter there was a rapid decrease, probably due to destruction of the 600 S component, but the activity was not completely lost, perhaps due to the survival of the 30 S component (Henle and Henle, 1947).

Flocculation

Flocculation occurs between immune serum and virus (Magill and Francis, 1938 *b*). The peak of flocculating activity develops in infected allantoic fluid after 48 hours (Henle and Chambers, 1941). From the work of Knight (see p. 589) with normal protein, it appears probable that flocculation may often involve normal constituents rather than virus antigens themselves No flocculation was found to take place when collodion particles, or bacterial cells, were mixed with influenza virus and exposed to immune serum (Pearson, 1944 *b*).

Antibody Absorption

Virus neutralizing antibodies can be absorbed (see, e g, Smith and Andrewes, 1938).

Burnet and his colleagues have worked on the absorption of neutralizing antibody by infected mouse lung or egg membrane (Burnet, Keogh, and Lush, 1937, Burnet, 1937 *b*, Burnet and Lush, 1938 *a*). They tested the immune body content of absorbed serum by different techniques. Using the chorio-allantois for the demonstration of antibody, they found that 90 per cent. could be absorbed with egg virus or mouse lung However, sera absorbed with egg virus showed no reduction in antibody when tested by inoculation of mice. Only by absorbing with large quantities of mouse lung was it possible to demonstrate reduction of antibody when tested by both chorio-allantoic and mouse inoculation They concluded that the apparent discrepancy in the result of titration by the 2 methods was due to the nature of the susceptible tissues, and not to any difference in the *in vitro* part of the reaction.

The antibody responsible for CCA-inhibition can also be absorbed from antiserum by treatment with an homologous virus preparation

Complement fixing antibody can also be absorbed, and this technique has been used in the study of the various components of influenza virus

Friedewald (1944 *a*, *b*) has made some studies on adsorption, testing for the presence of antibody by 3 different methods He used purified virus obtained from allantoic fluid as the absorbing agent, and human and ferret antisera After absorption, the sera were centrifuged and filtered through Gradocol membranes to remove virus. All sera were tested by the CCA inhibition and CF techniques, and some by inoculation of mice He found that the infectivity neutralizing, CCA inhibiting, and CF antibodies were completely absorbed by the homologous virus antigen, and that two absorptions were more effective than one The amount of antibody remaining unabsorbed varied inversely with the concentration of virus used to absorb A given concentration of virus absorbs a greater percentage of mouse-infectivity antibodies than either agglutinin inhibiting or complement fixing antibodies.

virus, vaccines derived from heterologous tissues proved less effective than those derived from homologous, as if foreign tissue interfered with the immunizing response. (3) The route of injection, intraperitoneal being more effective than subcutaneous. (4) Heat or formaldehyde decreased the immunizing potency of vaccines, especially those derived from heterologous tissues

Eaton (1940 *a*), working with mice, tested the relative immunizing value of active and inactive preparations Animals were inoculated by the peritoneal route, and tested after 14 days with an intranasal dose of between 1 and 10 MLD. He found that the average minimal immunizing dose of active PR8 virus was about 250 intranasal MLD, and of formolized virus the equivalent of about 8000 MLD.

It is questionable, however, whether purified formolized virus is less potent than material untreated with formalin Stanley (1945 *b*), using purified virus obtained by centrifugation, concluded that at equivalent dosages of virus material the immunizing potency for mice of formolized virus purified by centrifugation, formolized virus purified by adsorption and elution, and infective allantoic fluid were not measurably different.

SERUM ANTIBODIES IN EXPERIMENTAL INFLUENZA

Development after Active Immunization

Serum antibodies detected by various techniques develop in ferrets recovered from influenzal infection (Smith, Andrewes, and Laidlaw, 1933; Francis, 1934–5; 1935), and in ferrets subjected to vaccination with inactivated virus (see Andrewes and Smith, 1939).

Antibodies develop in the sera of mice after the inoculation of live or inactivated virus, and can also be found in the lung, antibodies persist longer in the serum of mice injected with live than dead virus (Oakley and Warrack, 1940). It has been suggested that in infected mice, the main site of production of antibodies is the mediastinal lymph glands (Burnet and Lush, 1938 *b*).

Antibodies have been described in the sera of the following animals after inoculation of live or inactivated virus rabbits (Francis and Magill, 1935 *b*, Fairbrother, 1938), hedgehogs (Stuart-Harris, 1936), pigs (see below); horses (Laidlaw *et al*, 1935; Burnet and Lush, 1938 *a*); guinea-pigs (Bijl and van Genderen, 1939, Burnet and Bull, 1943), monkeys (Burnet, 1941 *b*, Saslaw *et al*, 1946); Syrian hamsters and California kangaroo rats (Eaton and Beck, 1941).

The antibody response in animals is most specific on convalescence from infection, or following only one or two doses of vaccines. If the animals are hyperimmunized by repeated injections, the antibody response is no longer strain specific For the detection of antigenic differences between strains, therefore, convalescent sera, particularly ferret, are preferred (see, e g, Taylor and Dreguss, 1941; Friedewald, 1943)

Various observations have been made on the antibody level when partly immune animals are further exposed to virus Thus, when ferrets, in a state of partial immunity resulting from subcutaneous vaccination, or from the waning of a firm immunity after infection, are given an intranasal inoculation, there is an accelerated production of immune bodies, the titer rises much higher than after a primary infection Fully immune animals, however, show no further development of antibodies after a further injection (Francis, 1939).

Important studies of wide application have been carried out by McLean *et al.* (1945 *b, c, d*) in swine, on the effect of revaccination in immune animals, and on the relationship between antibody response and the dosage of virus inoculated. They used an allantoic fluid vaccine purified by centrifugation and inactivated by UVL or formalin

1 They found evidence of a definite trend towards an increased antibody response (measured by the CCA inhibition test), with an increase in dosage per unit

stance, egg-adapted virus inoculated intranasally in ferrets and mice stimulates resistance in 7–10 days (Burnet, 1937 *a*, 1938, Hyde and Chapman, 1937).

Virus cultivated in tissue culture can also be used to immunize (Magill and Francis, 1936, Rickard and Francis, 1938, Andrewes and Smith, 1939).

Vaccination with adjuvants. It has been found that the addition of certain agents, known as adjuvants, greatly increases the antibody response and the resistance to infection in animals injected with PR8, killed tubercle bacilli in paraffin oil and "Falba" base have been used (Friedewald, 1944 *a*). *Myco. butyricum* is probably more effective than the tubercle bacillus

Antibodies may be induced against antigenic material in the normal allantoic fluid (Friedewald, 1944 *c*).

Active Immunity Induced by Vaccines

Animals can be immunized effectively with influenza virus inactivated by various chemical and other agents. The immunizing potency of vaccines depends on the content of elementary bodies, and not on the amount of soluble substance present.

Elementary bodies heated at 57° C. were found to immunize mice and ferrets satisfactorily (Fairbrother, 1938, Fairbrother and Martin, 1939). Andrewes and Smith (1939) found that virus inactivated by heat was not as effective as living virus.

Formolized virus, either from mouse or ferret lung or allantoic fluid has been widely used, and stimulates resistance to infection and the development of antibody (Andrewes and Smith, 1939, Eaton, 1940 *a*, Oakley and Warrack, 1940). Formolized virus adsorbed on calcium phosphate is said to be more effective than unadsorbed virus (Salk, 1945). Excessive amounts of formol decrease immunizing potency (Stanley, 1945 *b*). Mice have been immunized with virus precipitated by chemicals such as alum (Bodily, Corey, and Eaton, 1943), and protamine (Chambers and Henle, 1941)

Virus inactivated by ultraviolet light can immunize mice on intraperitoneal injection, but is considerably less effective than live virus (Salk, Lavin, and Francis, 1940, Henle and Henle, 1947). Excessive exposure to UVL causes a loss in immunizing potency (Stanley, 1945 *b*). PR8 showed a gradual loss of immunizing activity on irradiation, although the HA titer was largely unaffected, after the HA test had become negative, the material still induced some immunity. With the Lee strain only prolonged irradiation reduced hemagglutination and antigenicity (Henle and Henle, 1947)

Virus inactivated by soap was found to be a much less effective immunizing agent than live virus (Stock and Francis, 1943)

Drying of formolized virus from the frozen state results in a loss of immunizing potency (Stanley, 1945 *b*)

Vaccines are usually given by the subcutaneous or intraperitoneal route Formolized virus has been given by repeated injections intranasally in mice (Lyons *et al*, 1944).

It may be mentioned here that mice immunized against various respiratory bacteria showed no increased resistance to the intranasal inoculation of virus A (Ungar and Hunwicke, 1941)

Relative Immunizing Effect of Live and Inactivated Virus Vaccines

It is generally agreed that inactivated preparations do not have such a high degree of *antigenic potency as preparations of live virus* (Andrewes and Smith, 1937, 1939, Hare, 1941, Eaton, 1940 *a*, Salk, Lavin, and Francis, 1940, Stock and Francis, 1943, Henle and Henle, 1947, McLean, Beard, and Beard, 1947)

Andrewes and Smith (1939) found that the immunizing efficiency of virus vaccines depended on (1) The titer of the contained virus (2) The source of the

5. Ferrets cannot be so easily protected as mice, and fever and nasal symptoms usually occur, although pulmonary involvement is frequently prevented (Zellat and Henle, 1941).

A curative effect of influenza serum can also be shown in experimental infection

1. If serum is given intranasally in mice up to 6 or even 24 hours after the virus, protection or reduction in the severity of lesions occurs (Henle, Stokes, and Shaw, 1941, Taylor, 1941 *b*).

The serum, or globulin fraction, can be given by spray (Lyons *et al*, 1944).

2. Serum given, even in large doses, subcutaneously or intravenously in mice is not as effective as small doses reaching the lungs after nasal instillation (Smorodintseff and Shiskina, 1940 *a*).

3. Hare (1939) found that the curative effect of serum could operate only if the infecting dose of virus was not much over 1 MLD

THE MECHANISM OF INFLUENZAL IMMUNITY IN THE EXPERIMENTAL ANIMAL

In discussing the question of why animals rendered resistant by vaccination or by recovery from infection are immune, 3 main mechanisms must be considered First the rôle of antibodies, second the importance of the reticulo-endothelial system, and third any changes that may occur in the respiratory tract rendering invasion less easy.

Generally speaking, the presence of serum antibody can be regarded as an indication of a degree of resistance to infection. For instance, Francis and Stuart-Harris (1938 *b*) found that immunized ferrets with serum neutralizing titers over 1/150 were generally resistant. A number of instances has, however, been recorded where animals with high antibody levels have succumbed to infection, and, on the other hand, where animals with low titers have proved resistant. Influenzal antibody can undoubtedly influence the spread of influenza virus *in vivo*, as it has been shown to have a powerful effect in passive immunity experiments It seems doubtful, however, whether influenza antibody, circulating in the blood, can prevent the entrance of virus into the superficially situated epithelial cells of the respiratory tract. This could be effected if antibody is secreted into the mucous secretions of the nose in the experimental animal, as in man (see p. 628), work in the rabbit suggests that this can occur in animals also. The rôle of circulating antibody is probably confined to preventing the spread of virus from the primary focus The experiments of Burnet in monkeys (1941 *b*) give some support to this conception. He found that when monkeys were infected on a number of occasions, by the intratracheal route, although the antibody level was high, a further rise occurred on each occasion. This suggested that the circulating antibody was unable to prevent invasion of the superficial epithelium, and that each antibody rise was due to a slight infection quickly overcome.

An important factor is probably the degree of rapidity with which antibody can be produced in response to infection. Experiments of Sugg and Magill (1946) on infection of actively immunized ferrets illustrate this point. They found that when ferrets with approximately the same antibody titers were inoculated with the same quantity of virus, the more resistant animals were those with the more extensive previous contact with the virus. The ferrets with the more extensive contact evidently more quickly mobilized a higher concentration of antibody. It is probable, therefore, that the protective effect of antibody is partly due to that amount present in the serum at the time of infection, and partly to what can be produced in response to infection

What evidence there is concerning the rôle of the reticulo-endothelial system, does not suggest that it plays any significant part in influenzal immunity. Thus Smorodintseff and Shiskina (1940 *a*) found that virus is not destroyed by the leukocytes of immune animals Virus injected intravenously is destroyed at the

weight. The relationship of the log. of the antibody titer to the log of the dose per 100 lbs. body weight was linear. The dose had, however, a relatively slight effect on the degree of antibody response. For instance, a tenfold increase in the dose of formolized virus gave only a 2 5-fold increase in titer. The increases in titer due to increases in dose were relatively negligible as compared to the degree of the antibody response to the smallest dose of virus.

2. They found the highest titer after vaccination occurred at 7 days, then there was a rapid decline, and the titer after 3 weeks was low. There was some indication that the rate of decline in titer was greater in those receiving the larger doses, and thus having higher titers. When formolized virus adsorbed on alum was used, the peak of the response was slightly delayed (1945 *c*).

3. Revaccination was carried out after 6 weeks, when there was again a definite indication of an increase in titer due to an increase in dose, but the dose had an even smaller influence on the titer attained after the second vaccination than the first.

A much greater rise in titer followed the revaccination than the first inoculation, and the titer declined more slowly.

4. They found that the optimum interval between doses of vaccine for keeping the titer at the highest level was 3 weeks.

Working with rabbits, Gaidamovich and Soloviev (1947) found the antibody content of lymph constantly related to that of blood, and usually representing about 60 per cent. of blood antibody. Only small amounts of antibody could be detected in the regional glands, and none in lymphocytes from thoracic duct lymph

PASSIVE IMMUNITY IN EXPERIMENTAL INFLUENZA

Antisera prepared by immunization of rabbits, ferrets, and horses with repeated doses of virus have definite protective and curative effects in influenzal infections of mice, the sera of ferrets recovered from infection are also effective (Francis and Magill, 1935 *b*, Laidlaw *et al.*, 1935, Hare, 1939, and others, see below) Mouse sera and human convalescent sera have also been used (Henle, Stokes, and Shaw, 1941), also the sera of guinea-pigs or rats injected nasally or intraperitoneally (Hyde, 1942)

The effective antibody is contained in the globulin fraction (see Lyons et al., 1944)

It should be realized in this type of work, that anaphylaxis may follow the repeated administration of serum Thus Hopps and Moulton (1943) found that guinea-pigs could be sensitized to foreign serum, and developed anaphylactic reactions when the "shocking" dose was given by inhalation.

The prophylactic effect of antiserum has been demonstrated in various ways

1. Serum administered nasally in mice protects against a subsequent instillation of live virus—even 100 or more MLD given 48 hours later (Vieuchange, 1940, Henle, Stokes, and Shaw, 1941, Taylor, 1941 *b*; Klein and Stevens, 1945). Serum will also protect against infection following inhalation of atomized virus (Wells and Henle, 1941)

2. The inhalation of immune serum is probably more effective than instillation (Zellat and Henle, 1941), and the degree of protection increases with the time of exposure to the spray (Lyons *et al*, 1944)

3. Serum given by routes other than the nasal, e g, subcutaneously, intravenously, or intra-abdominally is not so effective in preventing pulmonary infection in mice as serum given nasally (Hare, 1939, Smorodintseff and Shiskina, 1940 *a*, Henle, Stokes, and Shaw, 1941, Taylor, 1941 *b*)

4 Instillation of antiserum to one strain of virus A protects mice against infection with other strains of A, but not against infection with B virus (Hare, 1939, Zellat and Henle, 1941).

CHAPTER LXIII

IMMUNITY IN HUMAN INFLUENZA

ALLERGY

CERTAIN observations have been made suggesting that persons may become sensitized to influenza virus, so that on a later exposure they develop allergic manifestations. For example, Bull and Burnet (1943)[1] subjected volunteers to 2 exposures, by spray, of attenuated virus B at intervals of 3 to 6 months. Symptoms occurred as frequently after the second as the first exposure, and were more severe in those with high antibody levels. Virus was found fairly readily in the nasal secretion 48 hours after the first inoculation, but only rarely after the second. It is suggested that the symptoms were allergic in character.

In further work, Beveridge and Burnet (1944) found that unheated or boiled preparations of allantoic fluid infected with virus A or B might induce a skin reaction on intradermal inoculation In children the results of serum tests suggested that a positive skin test resulted from a previous infection with the corresponding virus. A number of children with positive serological tests, however, showed no skin reactivity. Most adults gave well-marked reactions, but the size bore no relation to the titer of serum antibodies About 20 per cent. of adults gave skin reactions to an antigen prepared from Newcastle virus, but this was probably due to a widening of the specificity of reactivity brought about by previous exposure to influenza virus

Henle *et al* (1946 *a*) in experiments on infection by inhalation found that there was some evidence that previous exposure to the virus resulted in a short incubation period, and that this was an allergic manifestation

ACTIVE IMMUNITY FOLLOWING NATURAL INFECTION

The resistance to reinfection following an attack of influenza is notoriously short. It must be realized, however, that in a proportion of cases where a second attack has followed a first after a short interval, the infecting agents may have been of different antigenic composition. Thus, the second infection may be due to a different strain of the same type (Magill, 1941), or to the other type of virus (see p 580)

ACTIVE IMMUNITY FOLLOWING EXPERIMENTAL INFECTION

It has been suggested that human beings can be actively immunized by the application of living virus to the nasal mucosa (Smorodintseff *et al*, 1937) Burnet and others have administered preparations of egg virus (A and B) of attenuated virulence for man by the nasal route; these preparations can be administered conveniently by spray (Burnet and Lush, 1938 *a*, Bull and Burnet, 1943; Mawson and Swan, 1943).

This type of immunization is now little used, partly because of the relative ease with which killed virus vaccines can be prepared, and partly because of a slight risk entailed by the inoculation of a living agent. Apart from the unknown susce ... e attenuated st ... lets ... 598), and a rise in antibody occurs (see p 667). A second exposure some months later usually gives rise to a less severe attack (Francis *et al*, 1944 *b*)

[1] References are appended at the end of this chapter.

same rate in organs rich or poor in reticulo-endothelial cells. Splenectomy or blockade does not markedly influence the resistance of normal or immunized mice

A number of observations has been made suggesting that resistance to reinfection *in immune animals is often brought about by* alterations in the physiological function or histological structure of the respiratory tract.

For example, working with mice inoculated intranasally with mouse-adapted strains, Oakley and Warrack (1940) found that the production of lung lesions was essential for the development of resistance to infection, and the production of antibodies. Straub (1939, 1940) concluded that resistance depends largely on the presence of epithelialized metaplastic areas in the bronchial tree; even slight metaplasia gives complete protection Eaton and Beck (1940) came to the same conclusion as Oakley and Warrack, when they used mouse-adapted strains. However, when they used tissue culture or ferret-passed strains, they found that mice became resistant without showing lung lesions. It has been reported that unadapted strains of human origin render animals resistant, and stimulate the production of antibodies without producing lesions in the lungs (Burnet and Clark, 1942).

That immunity can develop in mice without the presence of lung lesions, appears definitely established by the work of Rickard and Francis (1938). When mice were injected intraperitoneally with large doses of live virus, virus could be isolated from the lungs, being present in highest amount 48–72 hours later. These mice showed lung lesions, and were resistant to nasal inoculation. However, when mice were injected subcutaneously with live virus, or intraperitoneally with tissue culture virus, immunity was still produced, but no virus was found in the lungs

Important observations have been made in ferrets, which show that the production of the immune state after nasal instillation of virus is a complex process dependent on several factors (Francis and Stuart-Harris, 1938 *a*, *b*, Stuart-Harris and Francis, 1938). These authors found that ferrets, on recovery from infection, were resistant to nasal instillation of zinc sulfate, and to iontophoresis, for a short period This resistance appeared to be due to the altered histological structure of the nasal mucosa (see p. 600). It seemed, therefore, as if histological changes could account for the resistance to reinfection present in animals recently recovered from infection. When they investigated the effect of repeated nasal instillations of virus, they found the animals to be fully immune although the microscopic appearances of the mucosa were normal. In this case they attributed the immunity to the very rapid repair of the nasal mucosa, and to the development of antibodies.

There seems little doubt that both in ferrets and mice inoculated nasally both antibodies and histological changes are of importance in the acquisition of the immune state.

and the supernatant removed To elute virus, saline equal in volume to 1/10 that of the harvest material, was added to the cells The containers were incubated in a 37° C. water bath for 2½ hours. After low-speed centrifugation, the supernatant (containing the virus) was removed from the packed cells Formalin and merthiolate to give final concentrations of 1/2,000 and 1/20,000 respectively were added After 4 days, sterility tests were performed, and the bottles proving sterile pooled into lots containing equal quantities of PR8 and Lee viruses The efficiency of recovery of virus in the large scale process was over 70 per cent., and comparec [illegible] small scale

Hare and Mackenzie (1947) [illegible] red cell titration and antigenicity te [illegible] with falling dilutions and estimate tl [illegible] also Eddy, below).

Formolized vaccine obtained from allantoic fluids by adsorption to, and elution from, chick red cells was used in the extensive studies of the Commission on Influenza of the U.S Army (see below).

Beveridge and Burnet (1946) describe their technique for dealing with large numbers of eggs

McClelland (1946) has shown that typhus and influenza vaccines can be prepared from eggs infected in the yolk sac with the former agent, and in the allantoic cavity a few days later with influenza virus

5 *Centrifugation*, which has proved so effective in obtaining purified preparations of virus for laboratory experiments, can also be used for large scale production (Stanley, 1945 *b*, 1946, Taylor *et al*, 1945, Henle *et al*, 1946 *b*) For this purpose, the Sharples centrifuge is most suitable, Taylor *et al* (1945) recommend that the eggs be harvested in a chamber with UVL irradiation, and that the fluid be aspirated directly into a small harvesting flask. They used a modified type of centrifuge in which fluid is retained in the bowl during deceleration, to allow dispersion of virus aggregates by shaking, before withdrawal of the final concentrate, to "run through" 40 liters takes 20 hours, and gives an 800-fold concentration by volume The final product is a suspension of practically pure virus. They found that the yield of virus is about 90 per cent. or more of that initially present in the allantoic fluid

Stanley (1945 *b*) prepared vaccines containing 1-10 mgm. of virus per c c He found that vaccines treated with minimal amounts of formaldehyde or by exposure for a short time to UVL retained their full CCA activity and immunizing potency for mice Storage at 4° C. (but not higher temperature) resulted in maintenance of activity for at least 2 months To minimize the risk of bacterial contamination, he recommended the addition of 1/10,000 formalin and 1/100,000 phenylmercuric nitrate immediately after harvesting It was possible to prepare a vaccine by centrifugation containing 5 mgm Lee (B) and 2 5 mgm. each of Weiss and PR8 (A) viruses

Strains Included in Vaccines

In the earlier work it was usual to prepare the vaccine from one strain of virus only, and the WS strain was frequently used Owing to the discovery of antigenic variations between strains, and of the clear-cut differences between types A and B, it is now customary to use virus B and perhaps 2 strains of A. The strains chosen must not be highly specific, but should contain as wide a range as possible of the known antigenic components of the type. PR8 is probably the most popular A strain, and Lee of the B strains The Weiss strain of A has also been used to a considerable extent It is probable that a more recently isolated B strain should be used as well as the Lee (see Burner and Stone, 1946).

Assay of Vaccines

Various methods of assay have been used, usually depending on challenging mice, immunized with the vaccine under test, by the nasal route. These tests have not always proved satisfactory and it is now common practice in North America to rely more on the production of virus neutralizing antibodies in mice. For example, Eddy (1947) reported the following experiments carried out in attempting to formulate such a test

ACTIVE IMMUNITY FOLLOWING VACCINATION

Source of Vaccine

Older type of vaccine.

In earlier experiments, difficulty was encountered in obtaining a sufficient bulk of virus, free from foreign material. The types of vaccine that were used included

1. Formolized mouse lung, which produced a very satisfactory state of immunity in mice (Andrewes and Smith, 1937, Stuart-Harris, Smith, and Andrewes, 1940; Taylor and Dreguss, 1940 *b*).
2. Virus grown in tissue culture (Francis and Magill, 1935-6, 1937 *a*, Francis, 1937, Stokes *et al.*, 1937 *b*, Martin and Eaton, 1941; Siegel and Muckenfuss, 1941, Eaton and Martin, 1942, Siegel *et al*, 1942 *a*).
3. Virus grown on the chorio-allantois (Burnet and Lush, 1938 *a*).
4. Elementary bodies prepared by differential centrifugation of infected tissue, heated at 57° C. (Fairbrother, 1938).

The complex vaccine of Horsfall and Lennette.

These workers (1940 *a*) found that some ferrets experimentally infected with influenza developed distemper in convalescence A vaccine was prepared from their lungs and spleen, and given to stock ferrets. Subsequent tests showed that these animals were immune to distemper and influenza The influenzal antibodies persisted for a longer time than usual, and a marked rise was produced in human subjects also. The vaccine was difficult to prepare, and the beneficial effects could not readily be reproduced.

The vaccine was used in a group of 3500 persons, and appeared to give good protection against influenza A that broke out 4 months later, see Table 28 (Horsfall *et al*, 1941). The most effective vaccine in stimulating a rise in antibodies was one prepared from chick embryos infected with virus A, and the X strain of canine distemper (Horsfall, Lennette, and Rickard, 1941).

Results suggesting the protective value of this vaccine have been reported by others (Brown *et al*, 1941, Dalldorf, Whitney, and Rushin, 1941, Martin and Eaton, 1941, Eaton and Martin, 1942)

On the other hand, unsatisfactory results, as regards protection or antibody response, have been reported (Martin and Eaton, 1941, Hare, Auger, and McClelland, 1942, Hirst *et al*, 1942, Siegel *et al*, 1942 *b*)

Large scale production of vaccine from allantoic fluid.

Practically all vaccines are now prepared from allantoic fluid by various procedures, and this will be discussed in some detail, with particular reference to large-scale production

The main advantages of allantoic fluid vaccines are (*a*) The high degree of purification *and concentration of virus that is possible* (*b*) *The absence of risk of serious* contamination in preparation (*c*) As compared with the use of animals the egg is cheap (*d*) Concentrated preparations remain stable in the refrigerator for about a year (Salk *et al*, 1945 *b*). Allantoic fluid treated by various methods yields virus in sufficient quantity and concentration for the manufacture of vaccines

1. Alum-precipitated inactivated vaccine (Bodily, Corey, and Eaton, 1943)
2. Virus concentrated by *the freezing and thawing process* can also be used (Hirst, Rickard, and Whitman, 1942, Hirst, Rickard, and Friedewald, 1944) (see Table 28).
3. Henle *et al* (1946 *b*) have used a number of vaccines (*a*) Formolized and dialyzed fluids containing one or more strains (*b*) Allantoic fluid precipitated with protamine. (*c*) Formolized and dialyzed allantoic fluid dried by lyophilization They found that this process *caused some loss of immunizing capacity for mice*
4. Vaccine can be prepared by the *red-cell adsorption and elution process*, yielding a fluid concentrated about tenfold (Francis and Salk, 1942, Hare *et al*, 1943, Hare, Stamatis, and Jackson, 1943, Beveridge and Burnet, 1946, and see Table 28) Hare, Curl, and McClelland (1946) have reported on the value of this method for obtaining large quantities of virus A and B They inoculated 12-day eggs in the allantoic cavity with PR8 or Lee virus The eggs infected with PR8 were incubated for 48 hours at 37° C, 35° C being used for the Lee strain (It has since been found that 35° C. is optimal for both strains) In the process of harvesting, the chorio-allantoic vessels were intentionally torn, to allow bleeding into the cavity The harvested material was kept in the refrigerator overnight,

can be advanced to account for the failure to protect a number of persons in these experiments In certain cases the strain of virus included in the vaccine was found subse

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cases of so-called "Y" infection In other cases, the vaccine was given either too shortly or too long before the outbreak.

Doubtful Results

Other workers obtained results that could be considered as encouraging, yet without, for various reasons, affording clear-cut proof of the value of the vaccines used (Stokes *et al*, 1937 *b*, Salk *et al*, 1945 *b*, Friedman, 1946).

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Med. Lab, 1943 *c*,

Results of Vaccination against Experimental Exposure

Experiments have also been performed where immunized persons have been subsequently exposed to infection by atomized homologous virus Henle, Henle, and Stokes (1943) obtained satisfactory evidence of protection against virus A by this means, even 4 months after vaccination (see also Stokes and Henle, 1942).

Francis *et al* (1944 *a*, 1945) found protection against virus A after 2 weeks, but this had largely passed off after 4½ months.

A vaccine of virus B was also found to give satisfactory resistance to the homologous agent administered by spray (Salk *et al*, 1945 *a*).

Satisfactory Results

There has been a number of large scale vaccination campaigns where the reported results can be considered definitely encouraging, and statistically significant (see Table 28 for full details) The largest campaign so far reported is that of the Commission on Influenza of the U S Army.

Campaign of the Commission on Influenza.

The Commission on Influenza, U S Army, carried through a large scale experiment on the prophylactic immunization of several thousand troops The results were reported in a preliminary

later (Francis, 1945, Rickard,

1945 *a*, Eaton and Meiklejohn,

Menke, and Francis, 1945; Magill *et al*, 1945)

The vaccine was obtained from allantoic fluid by adsorption to, and elution from, chick red cells It contained strains PR8 and Weiss (A) and Lee (B), and was formolized Phenylmercuric nitrate was added as a bacteriostatic.

The vaccine was used in a single dose of 1 c c given subcutaneously. Controls received saline antiseptic solution only The vaccinations were begun in 6 different centers between October and early December 1943 A severe outbreak of influenza occurred in November.

The reports from the 6 centers, briefly, were as follows

1 At the University of Minnesota, the epidemic began 11 days after vaccination and the attack rate was 2 7 per cent. in the vaccinated group as compared to 9.06 per cent. in the controls The epidemic was proved to be due to virus A by isolation and serological response in a high proportion of the cases (Rickard, Thigpen, and Crowley, 1945).

2 In Iowa, Hale and McKee (1945 *a*) began their vaccination campaign at the beginning of the epidemic, and even so found a decreased incidence of the disease in the vaccinated group

1. Groups of mice received an intraperitoneal injection of test vaccine, neat, and *in varying dilutions. Two weeks later, a proportion of the mice was bled* from the heart, and the blood was pooled. Virus neutralization tests with this serum were carried out in mice by the nasal route. At the same time, the remaining immunized mice were challenged with an amount of virus equivalent to that used in the virus neutralization tests.

2. For the PR8 and Weiss (A) strains it required 63–125 times more vaccine to induce resistance, than to produce antibodies capable of neutralizing the same *amount of virus. For the Lee strain, approximately 5 times as much vaccine was* needed.

3. In some tests with virus vaccines, the end-point was taken as the greatest dilution of vaccine which produced immune serum capable of protecting 50–100 per cent. of mice against the challenge dose of virus A control vaccine showed an end-point for all 3 viruses of 1/125 or 1/625.

Dosage and Route of Inoculation

For the sake of convenience in mass inoculation campaigns, it is usual to give only one dose of vaccine. With modern purified vaccines a dose of 1 c c may contain up to 10 mgm. of virus (McLean *et al*, 1945 *d*, Stanley, 1945 *b*), in practice, however, very much smaller doses are used, owing to undesirable reactions with large amounts.

Although only one dose is usually given, it should be realized that as practically all adults have already been infected with influenza, this acts like a second or so-called "booster" dose. There is no doubt, that to keep the level of circulating antibody as high as possible, more than one dose should be given, particularly through the winter months.

Vaccines are usually given subcutaneously, but the intradermal and intramuscular routes have been used.

Complications of Vaccination

Certain workers have found quite a high incidence of local or systemic reactions after inoculation of vaccines Florman, Poindexter, and Council (1946) were unable to relate these reactions to initial antibody level, or to subsequent production of antibodies Reactions of this type are probably largely related to the virus content (Francis, 1947 *b*, *d*)

Ratner and Utrecht (1946), from a review of the literature and a study of a group of allergic children, conclude that serious and even fatal allergic manifestations may ensue in persons sensitive to egg white All hazard can be eliminated by the intracutaneous inoculation of 0 02 c c of undiluted vaccine before any prophylactic dose Those giving a moderate reaction should be given adrenalin along with the vaccine. Those giving a more severe reaction should receive the vaccine in divided doses Those giving a very severe response should not receive vaccine at all.

Bacterial Vaccines

It is not generally believed that bacterial vaccines have any effect in lessening the incidence of true virus influenza, although they may protect to some extent against upper respiratory disease (see, e g, Brady, 1942)

THE RESULTS OF VACCINATION IN THE PREVENTION OF INFLUENZA

Unsatisfactory Results

A number of workers has obtained definitely unsatisfactory results (see, e g, Andrewes, 1937, Stuart-Harris *et al*, 1938, Stuart-Harris, Smith, and Andrewes, 1940, Siegel *et al*., 1942 *a*, Hare, Stamatis, and Jackson, 1943) Various explanations

3. In California, Eaton and Meiklejohn (1945) did not find a significant difference in the incidence of the disease in the 2 groups The explanation probably lay in antigenic differences between the infecting virus and that used in the vaccine.

4 In New York, Hirst, Plummer, and Friedewald (1945) found a considerably lower incidence in vaccinated persons, although the epidemic started at the time vaccination was begun.

5. At the University of Michigan, Salk, Menke, and Francis (1945) completed vaccination about a week before the appearance of the first case. The incidence of clinical influenza in the vaccinated was 2 27 per cent. and in the unvaccinated 8 58 per cent. The authors believe that the benefits of vaccination may not be confined to the vaccinated, but confer some benefit on the unvaccinated members of the community also. The strain of virus responsible was closely related to the Weiss strain isolated earlier in 1943

6. In New York and Ithaca, Magill *et al.* (1945) found a significantly lower incidence among the vaccinated.

Experience in Great Britain

Vaccines were widely used in a controlled experiment in 1945–1946, but the subsequent incidence of influenza (B) was too restricted to evaluate the results accurately. Dudgeon *et al* (1946) suggested that there was some lessening of the incidence of infection with B virus. Stuart-Harris (1947) concluded that the vaccine used in these experiments appeared to benefit some large industrial groups, but not smaller communities. He raised the question of whether the benefit of vaccination *en masse* was due more to "herd immunity" than to resistance in the individual.

Conclusions

Certain of the above studies show that under favorable conditions, vaccination shortly before the onset or at the beginning of an epidemic can significantly lower the incidence of the disease, provided the infecting strain is closely related to the strain in the vaccine. The authoritative recent report of the Study Committee on Influenza Vaccination, American Public Health Association, points to the value of vaccination in certain circumstances, but does not recommend its general introduction (Francis, 1947*d*)

SERUM ANTIBODIES IN INFLUENZA INFECTION

In Children

The sera of infants contain antibodies to both swine and human viruses, these are presumably derived from the mother, as they disappear before about 4 months (Shope, 1936*a*, Burnet and Lush, 1938*a*, Rickard and Horsfall, 1941).

It is rare to find antibody in the sera of normal young children (Brown, 1936, Hare and Riehm, 1941) After the age of about 3, the percentage of normal sera containing antibody to human strains increases, but antibodies to swine virus have not been detected under the age of 10–12 (Andrewes, Laidlaw, and Smith, 1935, Shope, 1936*a*, Hare and Riehm, 1941). The antibody response of children to infection is usually highly specific Thus, Burnet and Lush (1938*a*) reported that when children's sera contained antibody, it neutralized only the homologous Melbourne strain Antibody to this strain was found in the sera of children born before the last epidemic, but not in those born later. Whereas absorption with swine virus removed all antibody from the sera of adults, it did not remove antibody to the Melbourne strain from a high titer serum of a child aged 3.

Hare and Riehm (1941) also found that in young children, following primary [illegible] bout 10,

(1945)

TABLE 28

LARGE SCALE CAMPAIGNS AFFORDING EVIDENCE OF THE VALUE OF VACCINATION AGAINST INFLUENZA

Authors	*Subjects*	*Source of Vaccine*	*Strains of Virus Included*	*Controls*	*Subsequent Exposure to Influenza*	*Incidence of Influenza*
Horsfall *et al* (1941)	3455 persons in institutions	"Complex" vaccine of influenza A and distemper viruses		4403 unvaccinated	Influenza A broke out 4 months later	Incidence in controls twice that in vaccinated group
Commission on Influenza (see *Commission*, 1944, Francis and colleagues, 1945, *Amer J Hyg 42*, 1–94)	approx 12,500 Army Forces (male)	Allantoic fluid concentrated by adsorption to and elution from chick r b c, formolized	PR8 and Weiss (A) Lee (B)	Alternate men received saline	Influenza A broke out towards close of immunization campaign	Attack rate 2 22 per cent in 6263 vaccinated men, and 7 11 per cent in 6211 controls
Hirst, Rickard, and Friedewald (1944)	3000 prisoners	Allantoic fluid concentrated by freezing and thawing, formolized	Usually PR8 (A) and Lee (B)	8000 uninoculated persons	Outbreak of "A" occurred one year later	Attack rate in vaccinated considerably lower. Average reduction of incidence 35 per cent
Francis, Salk, and Brace (1946)	600 Army Forces	As for Francis and colleagues (1945)		An unvaccinated group of 1000	Influenza B broke out following month	Incidence of hospital admission for acute respiratory disease 99 1 per 1000 unvaccinated and 11.5 in vaccinated group
Hirst *et al* (1947)	550 Army Forces at Yale University	As for Francis and colleagues (1945)		Unvaccinated group of 1050 cadets separately quartered	Influenza B broke out a few weeks later	Clinical influenza occurred in 12 5 per cent of unvaccinated controls, and 0.5 per cent of vaccinated men
Norwood and Sachs (1947)	366 industrial employees	As for Francis and colleagues (1945)		4280 workers in the same factory	Influenza B broke out almost at once	1 94 per cent of those vaccinated more than 7 days before developed influenza, 8 23 per cent of controls became infected

1941, Burnet *et al*, 1942, Eaton and Martin, 1942, Eaton and Meiklejohn, 1945, Magill *et al*, 1945, Rickard, Thigpen, and Crowley, 1945, Salk, Menke, and Francis, 1945).

For example, Hare, Auger, and McClelland (1942) examined the sera of 32 proven cases of influenza A in Toronto from 1937–1940, using a neutralization test with PR8 in mice. They found that the average initial titer of these patients was lower than the average for the population As far as could be determined, a titer of 1/60 appeared to be the lowest antibody level likely to be associated with resistance to infection In Australia, Burnet and Beveridge (1943) found that 18/19 cases of influenza had very low initial titers

In the Convalescent Phase of Influenza

The sera of convalescents from epidemic influenza usually neutralize the infectivity of human strains of virus in animal inoculation tests (Smith, Andrewes, and Laidlaw, 1933, Francis, 1934–5, Smith and Stuart-Harris, 1936, Francis and Magill, 1936, Smorodintseff *et al*, 1936 *b*, Brightman, 1936, Francis *et al*, 1937 *a*, Stuart-Harris *et al*, 1938, Brown *et al*, 1941, Hare and Riehm, 1941; Taylor and Dreguss, 1941, Sulkin, Smith, and Douglass, 1941, Hare, Auger, and McClelland, 1942).

Convalescent sera also fix complement in the presence of suitable antigens (Smith, 1936, Francis *et al.*, 1937 *b*, Hoyle and Fairbrother, 1937 *a*, Morrison *et al.*, 1939, Tulloch, 1939, Francis, 1940 *d*, Martin, 1940, Brown *et al*, 1941; Eaton, 1941, Eaton and Rickard, 1941, Foley and Burnet, 1941, Pearson *et al*, 1941; Sulkin, Smith, and Douglass, 1941, Hoyle, 1942, 1944, 1946) Antibodies to 600 S and 30 S develop separately in convalescence (Wiener, Henle, and Henle, 1946).

Antibody can also be detected by inoculation of virus-serum mixtures on the chorio-allantois (Burnet and Lush, 1940 *c*, Burnet, Cade, and Lush, 1940), or in the allantoic cavity (Burnet and Beveridge, 1943, Burnet and Stone, 1946).

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When the diagnosis of influenza is attempted by examination of an acute and a convalescent phase sample, every care should be taken that the first (acute) specimen is obtained within 5 days of onset, and the second (convalescent) at least 5 days after the first (see Florman and Crawford, 1944)

During the active phase, the antibody titer is usually low, but rises steeply after about a week to reach its maximum by the 14th day The antibody level rises higher in those with initially low titers than in those with higher initial titers For example, Francis *et al* (1937 *a*) found, by the mouse protection test, that the average *neutralizing titer of sera in the acute phase was 1/26, in convalescence*, the titer rose to 1/210 or even 1/640. The majority of cases in a series due to influenza virus A was found to have neutralizing titers of over 1/60 in convalescence, the average being 1/145 (Hare, Auger, and McClelland, 1942)

The complement fixation test may show a tenfold or greater increase in convalescence, for example, from 1/3.8 in the acute phase to 1/38 in convalescence (Sulkin, Bredeck, and Douglass, 1942), or 1/2 to 1 128 (Hoyle, 1946, see also Nigg *et al*, 1942). Hoyle (1944) regards fixation at a 1/16 dilution of serum as the sigr

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by the CCA-inhibition test. They found that the antibody response of children experiencing their first infection with virus A was greatest to the infecting strain and lower to PR8, WS, and swine strains. In late convalescence, the antibodies to swine virus completely disappeared.

The magnitude of the response, measured by the CCA-inhibition test, in infants presumably experiencing their first infection, has been found to be of the same order as that of older children (Adams, Thigpen, and Rickard, 1944).

One may liken the serological response to children to that of ferrets recovered from experimental exposure. The antibody rise is specific, being chiefly to the infecting strain; rises to related strains are slight. With increasing age, the reaction of the child resembles that of the adult

In Healthy Adults

It has been found that the sera of most normal adults, healthy at the time of test, and not recently convalescent from influenza, contain antibody to human strains of influenza virus. This antibody is generally believed to result from previous exposure, resulting in a clinical, or perhaps more frequently, a subclinical infection

Antibody to human strains has been detected by various methods in the sera of normal adults

1. By neutralization tests in animals (Andrewes, Laidlaw, and Smith, 1935, Francis and Magill, 1936, Brown, 1936, Fairbrother and Hoyle, 1937 *a*, Stuart-Harris *et al*, 1938, Tsurumi and Takagi, 1940, Burnet and Clark, 1942).

2. By neutralization tests in eggs (Burnet and Lush, 1938 *a*).

3. By the CF technique (Hoyle and Fairbrother, 1937 *a*, Lush and Burnet, 1937, Fairbrother and Hoyle, 1937 *a*, Tsurumi and Takagi, 1940, Friedewald, 1943).

4. The usual practice now is to titrate antibody by the CCA-inhibition test.

Antibody to swine virus also occurs in the sera of normal adults It is believed that this antibody is due to repeated exposure to human strains, as it were to hyperimmunization, and not to infection with the swine virus (Andrewes, Laidlaw, and Smith, 1935, Shope, 1936 *a*, Fairbrother and Hoyle, 1937 *a*, Burnet and Lush, 1938 *a*, Hare and Riehm, 1941; Kalter, Chapman, and Feeley, 1947). Virus neutralizing antibodies to swine virus have been found in placental globulin (Rake, 1939).

Certain observations suggest that, in nonepidemic times, random samples of serum from the community show a lower titer than is found immediately after epidemic periods (Stuart-Harris *et al.*, 1938, Martin, 1940).

St. Helena islanders. The island of St Helena escaped the great pandemic from 1917 to 1921, although influenza has occurred since that time Clearly, therefore, the study of sera might throw interesting light on the possible rôle of the swine influenza virus in the pandemic Stuart-Harris *et al* (1938) studied the sera of 23 islanders, these persons had never left the island and were alive in 1918 Only one serum had fair neutralizing antibody to the WS strain, and one serum to the swine strain. No influenza had occurred in the island for 6 years at the time of this test, but shortly afterwards, influenza happened to break out Twenty-two of the original sera were reexamined (only a few months after the first test), and many now showed a significant level of antibodies to both WS and swine virus This observation does not suggest that the presence of immune bodies to the swine virus need imply any specific contact with that agent

In the Acute Phase of Influenza

It seems reasonably well established that a low initial titer of antibody is a factor in determining susceptibility to infection Samples of serum removed from persons in the early stages of influenzal infection frequently show somewhat lower titers of antibody against the infecting strain than are usual in a normal section of the community at the time (Hoyle and Fairbrother, 1937 *a*, Stokes *et al*, 1937 *a*, Stuart-Harris *et al*, 1938, Rickard, Lennette, and Horsfall, 1940, Rickard *et al.*,

1941; Burnet *et al*, 1942; Eaton and Martin, 1942, Eaton and Meiklejohn, 1945; Magill *et al*, 1945, Rickard, Thigpen, and Crowley, 1945, Salk, Menke, and Francis, 1945)

For example, Hare, Auger, and McClelland (1942) examined the sera of 32 proven cases of influenza A in Toronto from 1937–1940, using a neutralization test with PR8 in mice. They found that the average initial titer of these patients was lower than the average for the population As far as could be determined, a titer of 1/60 appeared to be the lowest antibody level likely to be associated with resistance to infection In Australia, Burnet and Beveridge (1943) found that 18/19 cases of influenza had very low initial titers

In the Convalescent Phase of Influenza

The sera of convalescents from epidemic influenza usually neutralize the infectivity of human strains of virus in animal inoculation tests (Smith, Andrewes, and Laidlaw, 1933, Francis, 1934–5; Smith and Stuart-Harris, 1936, Francis and Magill, 1936, Smorodintseff *et al*, 1936 *b*, Brightman, 1936, Francis *et al*, 1937 *a*, Stuart-Harris *et al*, 1938, Brown *et al*, 1941; Hare and Riehm, 1941, Taylor and Dreguss, 1941; Sulkin, Smith, and Douglass, 1941; Hare, Auger, and McClelland, 1942)

Convalescent sera also fix complement in the presence of suitable antigens

separately in convalescence (Wiener, Henle, and Henle, 1946).

Antibody can also be detected by inoculation of virus-serum mixtures on the

1946).

When the diagnosis of influenza is attempted by examination of an acute and a convalescent phase sample, every care should be taken that the first (acute) specimen is obtained within 5 days of onset, and the second (convalescent) at least 5 days after the first (see Florman and Crawford, 1944).

During the active phase, the antibody titer is usually low, but rises steeply after about a week to reach its maximum by the 14th day The antibody level rises higher in those with initially low titers than in those with higher initial titers. For example, Francis *et al* (1937 *a*) found, by the mouse protection test, that the average neutralizing titer of sera in the acute phase was 1/26, in convalescence, the titer rose to 1/210 or even 1/640 The majority of cases in a series due to influenza virus A was found to have neutralizing titers of over 1/60 in convalescence, the average being 1 145 (Hare, Auger, and McClelland, 1942).

The complement fixation test may show a tenfold or greater increase in convalescence, for example, from 1/3 8 in the acute phase to 1/38 in convalescence (Sulkin, Bredeck, and Douglass, 1942), or 1/2 to 1/128 (Hoyle, 1946, see also Nigg *et al*, 1942) Hoyle (1944) regards fixation at a 1/16 dilution of serum as the significant level for diagnostic tests (see also Hoyle and Fairbrother, 1947)

inhibition test, is diagnostic (Beveridge and Williams, 1944; *Burnet, Stone, and* Anderson, 1946).

A number of observers has studied the rise and subsequent fall in antibody titer of convalescents over a prolonged period (Francis *et al.*, 1937 *b*; Fairbrother and Martin, 1938; Tulloch, 1939, Horsfall, Hahn, and Rickard, 1940; Eaton and Rickard, 1941, Horsfall and Rickard, 1941). The maximum titer is usually reached between the 10th and 14th day. A decline in titer begins between the 11th and 25th day and continues progressively. The higher the maximum titer, the quicker the decline. By about 2–5 months after an attack, the titer is usually about half that of the maximum. The titer declines slowly, and by about 10–12 months after the attack, is back to its original preinfection level.

Schwartz *et al.* (1946) have shown that examination of pooled sera from the general population after an epidemic shows a high antibody level, and that this falls in a few months

A rise in antibody to swine virus usually accompanies an increase in antibody to human strains (Hare and Riehm, 1941; Horsfall and Rickard, 1941, Bodily and Eaton, 1942; Sigurdsson and Bjarnason, 1946). The titer against swine virus may often be higher than that against human strains (Hare and Riehm).

The antibody response in infected human beings is usually wide, and not strain specific (see Taylor and Dreguss, 1941). For example, the antibody response to infection with virus A was studied in 5 cases of acute influenza by Horsfall and Rickard (1941) using the neutralization test in mice. There was an almost complete lack of strain specificity in the response, and a more or less equal rise and subsequent fall in the antibody against several A strains and the swine virus As the response is not usually strain specific, Magill and Sugg (1944) recommend the use of several strains in neutralization tests. The antibody rise in convalescence is usually type specific. Antibody to B does not, as a rule, rise significantly in A cases, such an occurrence has been described by Bodily and Eaton (1942), who advance the hypothesis that the rise may be in the nature of an anamnestic reaction due to previous infection with virus B A rise in antibody to A virus may occur in B infections (Dudgeon *et al.*, 1946)

In some cases the sera may show a much more marked reaction with the infecting strain that with a stock strain of the same type of A or B virus (Eaton and *Meiklejohn, 1945, Salk, Menke, and Francis, 1945, Dudgeon et al., 1946*). Examining convalescent sera in an epidemic of B infection Burnet and Stone (1946) found that adolescents tended to show a specific response, the rise to the homologous strain being greater than that to stock Lee virus. In adults, on the other hand, the response tended to be nonspecific, in that rise to Lee was greater than that to the current strain These differences were not absolute

Although a rise in antibody, of varying degree, practically always occurs in influenza, it is important to realize that definite cases of the disease *may show no* rise at all (Magill, 1940, Francis *et al*, 1945, Burnet, Stone, and Anderson, 1946, Dudgeon *et al*, 1946)

Antibody neutralizing the toxic effect of influenza virus develops in convalescence (Henle and Henle, 1946 *b*)

In Subclinical Infection

Sampling of numerous sera has shown that a proportion of persons *exposed to* influenzal infection develops a rise in antibody [illegible] techniques, without having had any clinical eviden[illegible] fection has been recorded with virus A (Franci[illegible] *et al.*, 1938, Burnet and Lush, 1940 *c*, Burnet, Cade, and Lush, 1940, Rickard, [illegible] nette, and Horsfall, 1940, Nigg *et al*, 1942, Horsfall, Hahn, and Rickard, 1940, Siegel *et al.*, 1942 *a*, Crowley, Thigpen, and Rickard, 1944, Salk, Menke, and Francis, 1945, Magill *et al*, 1945)

Subclinical infections have also been reported due to virus B (Salk *et al*, 1945 *b*, Burnet, Stone, and Anderson, 1946).

In Experimental Infection

Persons exposed experimentally by nasal instillation or inhalation of virulent or attenuated virus (A or B) develop an increase in antibody, a not inconsiderable proportion of these persons shows no symptoms, evidently suffering from a subclinical attack (Smorodintseff *et al*, 1937, Burnet and Lush, 1938 *a*, Burnet and Foley, 1940, Francis, 1940 *c*, Foley and Burnet, 1941, Bull and Burnet, 1943, Burnet and Bull, 1943, Burnet, 1943 *b*, Mawson and Swan, 1943, *U.S. Nav. Med Lab.* 1944 *b*, Henle *et al*, 1946 *a*, Wiener, Henle, and Henle, 1946).

Most clinical cases of influenza following experimental exposure occur in the group of subjects with the lowest antibody titer, although there are many excep-

vac-

irus,

was

high or low (Salk *et al*, 1945 *a*, Henle *et al.*, 1946 *a*).

The administration of a second dose of live virus (B) 4 months later, was found to give another rise in antibody, although it was not so high as in those sprayed for the first time (Francis *et al*, 1944 *b*).

Correlation of Antibody Titers Determined by Various Methods

It is generally agreed that there is a close qualitative correlation between the results obtained by the various methods for the detection of antibody. That is to say that if antibody is found by one method it will usually be found by others as well, if antibody shows a significant rise in convalescence when tested by one method, it will show a rise similar in magnitude when tested by another method Some of the observations that have been made comparing various tests are as follows

The development of virus neutralizing, CF and flocculating antibodies in acute and convalescent sera was found to show good agreement (Henle and Chambers, 1941)

In general, there is good agreement between the results of virus neutralization tests conducted by the CCA-inhibition technique and by intranasal inoculation in mice (McClelland and Hare, 1941; Bodily and Eaton, 1942, Henle, Henle, and Stokes, 1943, Burnet *et al*, 1945)

Hirst (1942 *c*) investigated this problem, using a number of human and ferret

ization and complement fixation with 600 S; there may be a different antibody for fixation with 30 S (Wiener, Henle, and Henle, 1946).

The Significance of Antibody to the Swine Virus in Human Sera

There is a wide distribution of immune bodies to the swine influenza virus in adult human sera, and there are 2 alternative theories advanced to explain their presence.

1. It has been suggested that the virus of swine influenza *represents the human* strain that caused the 1918 pandemic (Laidlaw, 1935; Andrewes, Laidlaw, and Smith, 1935, Shope, 1936 *a*). That is to say, that the human pandemic strain does not now attack man, but only pigs, and the present human virus is a variant of the original. The evidence upon which this theory was based is as follows:

(*a*) There has been no later pandemic of human influenza in any way comparable to that of 1918. At the present day there does not appear to be any influenza virus, attacking man, of such virulence as was manifested in 1918.

(*b*) Swine influenza broke out at the time of the pandemic, and many persons thought that they were one and the same condition. Swine influenza has not died out, and the virus is still being propagated under natural conditions

(*c*) The properties of the swine and human influenza viruses, as isolated today, are virtually identical. They can, however, be differentiated antigenically.

(*d*) The distribution of antibodies to human and swine influenza viruses in the human serum at different ages may be important in this connection. Thus the majority of adults, and a slightly lower number of children's sera neutralized the WS strain (Andrewes, Laidlaw, and Smith, 1935). Antibodies which neutralized the swine virus were regularly present in the sera of adults, but were absent from the sera of children under 10. Further, Shope (1936 *a*) in the United States found *swine influenza antibodies to be absent from sera of children below 12.*

It appeared from these observations that adults had been exposed to the swine influenza virus presumably during or prior to the 1918 pandemic, and had developed immune bodies. Children, however, born since the pandemic, had not been so exposed and had only developed immune bodies to the prevalent human type of virus The main basis for this theory was the demonstration of immune bodies in the sera of adults, while absent from children's.

2. The alternative view, that the presence of antibody to swine virus can be explained with reference to the human strain alone, was expressed by Burnet and Lush (1938 *a*) The following facts can be adduced in support of their contention which is now accepted as being almost certainly correct

(*a*) The swine and human influenza viruses share a common antigen (see p 587).

(*b*) *Hyperimmunization with the human virus in animals results also* in the production of antibodies to the swine virus (Francis and Shope, 1936, see p. 594).

(*c*) Horse antiserum prepared by injection of WS virus acts on swine virus as well (Burnet and Lush, 1938 *a*)

(*d*) During convalescence from influenza contracted from a ferret infected with human virus, Stuart-Harris developed immune bodies to both strains of virus (Smith and Stuart-Harris, 1936).

(*e*) Convalescents from influenza on St Helena, who had never been exposed to the 1918 pandemic, developed antibodies to both strains (Stuart-Harris *et al*, 1938).

(*f*) The serum of children is poor in antibody, but what there is proves highly specific and cannot be absorbed by swine virus (Burnet and Lush, 1938 *a*) These children have probably had only slight contact with human virus and no hyperimmunization *has resulted, hence the specificity*

(*g*) Adult serum contains antibody acting on both human and swine viruses Although of high titer following recovery from infection, the level soon falls In the majority of adult persons the influenzal antibodies appear to be not highly specific, and can be absorbed with swine virus

Antibody to swine influenza in human sera does not appear to indicate contact with that particular virus, but is a group antibody produced by repeated contact and hyperimmunization with the human virus, which possesses a common antigen

PASSIVE IMMUNITY IN HUMAN INFLUENZA

It has been claimed that single or repeated inhalations of 2–3 c.c. of atomized antiserum reduces the incidence of influenzal infection (Smorodintseff, Gulamow, and Tschalkina, 1940).

Stuart-Harris *et al.* (1938) injected large doses of serum prepared in the horse, but were unable to say that the results obtained were specific.

SERUM ANTIBODIES FOLLOWING VACCINATION

Significant rises in antibody after vaccination were found by the earlier workers, and the antibodies were shown to persist for at least 5 months (Francis, 1937, Francis and Magill, 1937 *a*).

This question has been extensively studied of recent years, and the following are the more important observations

1. There is general agreement that the same information as to the presence of antibody and the extent of its rise subsequent to vaccination can be obtained by any of the usual tests. The CCA-inhibition test, however, is now most used (see [illegible]

2. There is a wide individual variation in the response of human beings to the same preparation (Hirst *et al*, 1942). While the majority will show a fourfold or greater rise to one of the components of the vaccine, it is unusual for antibodies to develop to all components equally (see Florman, Poindexter, and Council, 1946).

3. The best antibody response occurs in those with an initial low titer, those with a high titer showing only slight rises (Eaton and Martin, 1942, Magill *et al.*, 1945; Rickard, Thigpen, and Crowley, 1945, Salk, Menke, and Francis, 1945).

4. The maximum rise in antibody occurs in 10–14 days, and then begins to decline One can expect antibody increases of 6–9 fold 2 weeks after inoculation. Even after 2–3 months there is a considerable drop Persons developing the highest titers tend to show the most rapid decline After 6–9 months the titer may be from ¼ to ½ of the maximum, and remains above the initial level for about a year (Eaton and Martin, 1942, Hirst *et al*, 1942; Henle and Henle, 1945 *a*, Magill *et al*, 1945, Salk, Menke, and Francis, 1945, Salk *et al.*, 1945 *b*) For example, using a concentrated vaccine (freeze-thaw process) the mean antibody rise detected by CF and CCA-inhibition was 6-fold at 2 weeks, and 3-fold about a year later (Hirst, Rickard, and Friedewald, 1944).

There may be a marked negative phase a few days after injection (Hare *et al*, 1947).

5. Antibody rises produced by potent vaccines given subcutaneously are as marked as follow natural infection (Hirst *et al*, 1942, Hare *et al*, 1943; Rickard, Thigpen, and Crowley, 1945)

Although it is unwise to be too definite, if a titer of over 1/128 in the CCA-inhibition test develops after vaccination there is a considerable likelihood of protection (see Henle *et al*, 1946 *b*)

6 The actual physical amount of virus injected in the vaccine is the main factor that determines the height of the antibody response, apart from variations in the person injected, the mean geometric antibody response increases parallel with the amount of virus injected (Hirst *et al.*, 1942, Henle *et al.*, 1946 *b*). The degree of antibody response in man produced by a vaccine is highly correlated with immunizing capacity for mice (Henle *et al.*, 1946 *b*)

Better responses are obtained, therefore, when concentrated vaccines are compared with diluted preparations or with simple allantoic fluid (Hirst, Rickard, and Whitman, 1942, Hare *et al*, 1943, 1947, Hirst, Rickard, and Friedewald, 1944).

It appears that much the same effect can be produced by giving a larger dose (e.g., 3 c.c.) of simple allantoic fluid (Henle *et al.*, 1946 *b*).

7. The effect of inactivation of virus on antibody response has been studied. Thus Hirst *et al.* (1942), using allantoic fluid, found that similar titers were obtained after inoculation of active virus, formolized virus, heated virus, or dried inactivated virus

They found that a vaccine composed of ground infected embryos diluted with infected allantoic fluid gave a greater response than allantoic fluid only, but the antigenicity of such a preparation was decreased by formolization.

8. The antibody response of vaccinated persons is not strictly strain specific, resembling in this respect the response of infected adults. There is some suggestion that the response after vaccination may be more specific than that following infection (see Bodily and Eaton, 1942).

9. As regards the effect of revaccination, those who develop a significant rise in antibody mostly fail to develop a further rise after a repeat injection given within a few weeks (Beveridge, 1944 *b*, Salk *et al*, 1945 *a*). This may be explained by suppression of the antigenic properties of the second injection by circulating antibody (Beveridge, Stone, and Lind, 1944).

There does not seem to be any advantage in giving more than 2 injections within a short interval, as the geometric mean antibody levels are not increased thereby (Hare *et al.*, 1943; Henle *et al*, 1946 *b*).

Revaccination after a lapse of some weeks will cause a further rise in antibody level. By repeated injections every 6–8 weeks, it should be possible to maintain a high level of antibody throughout the winter months. (See the observations of McLean *et al.*, 1945 *d*, on revaccination of swine)

10 It has been reported that vaccinated persons who become infected during the period when their antibody is falling, show a secondary rise, if infected in the early stages, while the antibody titer is increasing, the rise is prolonged (Siegel *et al.*, 1942 *a*).

11 Occasionally, inoculation of potent vaccines may fail to stimulate a demonstrable rise in antibodies (Stokes *et al*, 1937 *a*). Later observations have shown that this usually occurs only in those with high initial titers. Some persons who fail to develop antibody to the strains included in the vaccine may do so to related viruses (Florman, Poindexter, and Council, 1946).

12 It has been shown that the rise in antibody titer after 1 intradermal injection is considerably higher than after 2 such injections, or 1 subcutaneous injection (Van Gelder, Greenspan, and Defresne, 1947).

Serum antibodies after use of "adjuvants."

Henle and Henle (1945 *a*) compared the antibody responses in subjects vaccinated with virus A and B in saline, and the same agents emulsified in "Falba" and mineral oil In the persons vaccinated with adjuvants, the antibody titers rose more slowly than usual to reach a peak after 3 months, when they were about 5 times as high as those in the virus-saline group Even after 12 months, the levels in the adjuvant group were still above the maxima reached by the controls, and the majority of persons was probably protected (Henle *et al.*, 1946 *b*).

MECHANISM OF INFLUENZAL IMMUNITY IN HUMAN BEINGS

It is still imperfectly understood what mechanisms underlie resistance to influenza, but the work of the last 10–15 years has clarified many aspects of the problem (see in particular, Francis, 1941–2) Obviously both the parasite and the host are concerned

As regards the influenza virus, there are 2 distinct antigenic types, A and B, which give rise to no cross immunity An attack of influenza A will not immunize against influenza B. Second attacks of influenza at a short interval may well, there-

fore, be due to infection with a different type, and this has actually been described (see p 580). Antigenic variations within the type may be considerable, and it is not impossible that a second attack may be due to another strain of the same type. Then there is the question of so-called influenza Y (see p. 580), which may perhaps be due to a third type of virus, as yet unisolated, and antigenically distinct from virus A or B.

Andrewes has postulated the existence of influenza viruses of various grades of virulence (see p 583), and it certainly appears that the clinical syndrome of influenza can be caused by agents of widely different pathogenicity to ferrets and mice. It can be suggested that an attack of influenza due to a virus of low grade virulence would not immunize against attack by a virus of virulence "stepped-up" by frequent transfer. It has been suggested that influenza virus may elaborate a toxin, and possibly this factor is of importance in determining the virulence of a strain (see p. 608).

As regards the host, it is generally agreed that practically every person becomes infected with the virus at a fairly early stage in their life Antibodies to the virus are rare in early childhood, but are almost universal in adolescence and adult life (see Ch. LXIII)

[illegible]

conferring immunity if the antibody is raised above a certain level (see p 669).

When a person with antibody in the blood becomes exposed to reinfection, it is probable that invasion of the superficial respiratory epithelium, i e, in nose, trachea, and perhaps bronchi, occurs. Circulating antibody cannot prevent this invasion, but it probably can limit the further spread of the agent The invasion of virus acts as a further stimulus to the production of fresh antibody.

The nasal secretion of certain persons contains a virus inactivating agent which is probably serum antibody (see p. 628) An agent like this could presumably protect superficial cells from invasion, and could limit spread of the virus from cell to cell.

Reinfection under experimental conditions is often associated with some symptoms in the upper respiratory tract, and it may be that these are allergic in character (see p 657), and represent the reaction of the sensitized tissues of the upper respiratory tract to the specific agent.

In animals, recovery from infection is associated with histological changes in the nose (ferret) or respiratory tract (mice). These changes appear to confer a certain degree of immunity to reinfection (see p. 655). It is not known whether such changes occur in man, but it seems possible.

In conclusion, therefore, it is evident that influenzal immunity in man is complex The presence of antibody in blood and nasal secretion is the most important factor as regards the host, allergy, and structural changes in the respiratory tract, may also be of importance. As regards the parasite, antigenic structure and virulence are the factors that determine its probable pathogenicity for any given person

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HISTOLOGY

Hogan and Crawford (1942 *b*) suggest that the lesions in the cornea are in the nature of an acute dilatation and swelling of the corneal lymphatics, with edema of the affected tissues. Lymph spaces become filled with debris. In severer cases, there is actual destruction of tissue

Feigenbaum, Michaelson, and Kornblüth (1945) described a flattening of the corneal epithelium, marked capillary dilatation, and edema in the subepithelial tissue. There were subepithelial infiltrations with lymphocytes and large mononuclears Mitoses were seen. In chronic cases, hypertrophied papillae were present.

EPIDEMIOLOGY

Age incidence. All ages are susceptible, and cases have been seen in infants

Geographical incidence. The disease has a widespread distribution A number of severe outbreaks of the disease have been reported from India (Herbert, 1901; Kirkpatrick, 1920; Wright, 1930, Kirwan, 1935). It also occurs in Malay, Java, China, and Japan (see Bedell, 1943), and in Tasmania (Hamilton, 1942).

In Palestine, Feigenbaum, Michaelson, and Kornblüth (1945) studied nearly 400 cases The disease was most prevalent at the beginning of the rainy season. At first the cases were in coastal areas, but later were reported inland.

in the Second World War, mainly in shipyards and industrial plants, occurred in the San Francisco Bay area from September 1941 to January 1942 (Hogan and Crawford, 1942 *a, b*, Kuh, 1943). Portland (Oregon) was visited in November and December 1941 (Rieke, 1942). Cases were also seen in Seattle (see Bedell, 1943). Later, in 1942, cases were seen on the eastern seaboard in New York, Schenectady and inland (Perkins, Korns, and Westphal, 1943; Sanders and Alexander, 1943; Sanders *et al.*, 1943, Korns, Sanders, and Alexander, 1944) An outbreak in Detroit was described by Molner and Cooper (1944). The features of the disease in recent American outbreaks have been fully described by the above authors and other sources (Circular Letter No 14, 1943; *J. Amer. med. Assn*, 1943).

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Occupational incidence. Although most of the recent American cases occurred in industrial workers, such as shipyard employees, many nonindustrial workers were affected It is probable that the conditions of work in industrial plants are often favorable to the spread of the virus of EKC The incidence of attack in industrial plants has usually been about 5 per cent.

Method of spread There seems little doubt that minor traumata, such as the presence of noxious gases or dust, predispose to infection. The disease may be spread by direct contact with conjunctival secretion, or by means of fomites such as soap, towels, and washbasins The infection may be passed through touching the hands of a sufferer, and transferring the infection to the eye by rubbing

Equipment used in examining and treating eyes may transfer the infection, and on no account should eye-droppers or brushes be returned to a bottle of lotion before being sterilized Physicians or nurses may inadvertently infect themselves unless vigorous handwashing is performed after attending to cases (see Allen, 1944).

SECTION 6. VIRUS INFECTIONS OF THE EYE

CHAPTER LXIV

EPIDEMIC KERATOCONJUNCTIVITIS

HISTORICAL

EPIDEMIC keratoconjunctivitis was *probably first described by German workers at* the end of last century (Fuchs, 1889; von Reuss, 1889, von Stellwag, 1889). Since then it has been reported from India, the Far East, Britain, America, and elsewhere. The clinical features of the disease are quite characteristic, and suggest an infective origin. Epidemic keratoconjunctivitis has for long been held to be a virus infection on general grounds, but only quite recently has a specific filtrable agent been described (Sanders, 1942; Sanders and Alexander, 1943).

It is probable that epidemic keratoconjunctivitis (EKC) is identical with a number of other conditions called variously. superficial punctate keratitis, keratitis subepithelialis, keratitis maculosa, and nummular keratitis. The condition known as Béal's conjunctivitis may be similar It is not, however, possible to be quite certain of the identity of these conditions with EKC, until virus studies have been carried out. We shall confine our description to the features, epidemiology, and etiology of "epidemic keratoconjunctivitis," which has all the characters of a specific infection, with particular reference to recent American experience. Full reviews of the literature are given by Hogan and Crawford (1942 *b*), Bedell (1943), and Rados (1944).

CLINICAL FEATURES

The incubation period of the disease is about 5–10 days.

There may be a short prodromal period characterized by malaise and slight pyrexia. The onset of conjunctivitis is sudden, and there is usually a feeling as if a foreign body is present. In fact, most patients seek attention for this complaint. There is hyperemia and edema of the palpebral and bulbar conjunctivae. The lids, chiefly the upper lid, are edematous The sclera is congested. There is usually a watery discharge from the affected eye, and photophobia. Pseudomembranes may form, usually in the lower lid, on removal these leave bleeding points Petechial hemorrhages may be found in the conjunctiva

The cornea is involved in 50–90 per cent. of cases, from 1–5 weeks after onset. *Discrete grayish infiltrates develop in the epithelial and subepithelial layers,* usually in the pupillary area The overlying epithelium is seldom ulcerated

A very characteristic feature is the presence of enlarged tender preauricular glands, sometimes other glands are involved also.

The disease usually begins in one eye, and in about half the cases the other is *involved after a few days, the disease tends to run a shorter* and milder course in the second eye.

EKC is of considerable economic importance, as sufferers are unable to work for some time Ocular sequelae, however, are unusual

Microscopic examination of the exudate shows the presence of mononuclear cells. No *definite inclusions have been seen Only the usual conjunctival* flora are found on bacteriological examination, sometimes the cultures prove sterile.

Laboratory diagnosis is made by inoculating tissue cultures or mice with conjunctival exudate or fragments of corneal tissue (see below)

5. The Virus of Sanders and Alexander

There seems little doubt, although the virus was isolated on only a few occasions, that the cause of the recent American outbreaks was a virus isolated by Sanders (Sanders, 1942, Sanders and Alexander, 1943, Sanders *et al*, 1943). The virus was isolated from conjunctival fragments in tissue cultures, and was adapted to mice Braley (1945) confirmed these observations Another strain of this type of virus was isolated by Maumenee, Hayes, and Hartman (1945).

Animals.

The virus can be passed to adult *mice* by the intracerebral or intranasal routes, or to unweaned mice by the peritoneal route (Sanders, 1942, Sanders and Alexander, 1943, Sanders *et al*, 1943, Braley, 1945). The symptoms develop after 48–72 hours, are not severe, and the animal usually recovers Virus is found mainly in the brain. Histological changes are slight

On intracerebral injection, Sanders' virus may produce sudden death on the 8th day in rabbits (Sanders and Alexander, 1943)

Feigenbaum, Michaelson, and Kornblüth (1945) isolated a virus similar to that of Sanders, and found that it produced opacities in the rabbit cornea 24–48 hours after inoculation which might persist for several weeks

Filtration and cultivation.

[illegible] -feld filter, it passes through mem- [illegible] through those of 50–75 mu APD [illegible] ichaelson, and Kornbluth (1945) also found the virus to be filtrable

Virus in conjunctival scrapings can be propagated in embryo mouse brain serum ultrafiltrate cultures (Sanders and Alexander, 1943, Sanders *et al*, 1943, Braley, 1945, Feigenbaum, Michaelson, and Kornbluth, 1945). Virus proliferates best at room temperature Transfers must be made with ground up cells. The presence of virus is shown by inoculating mice intracerebrally.

After inoculation in the allantoic cavity, the embryo died in 5–6 days. Passage can be continued and the allantoic fluid infects mice (Calkins and Bond, 1944)

Relationship to other viruses.

The virus appears to be specific, and Sanders and Alexander (1943) found that their agent was not neutralized by antiserum to LCM or herpes viruses Mice immunized against Theiler's virus were susceptible to EKC virus. EKC virus was not neutralized by hyperimmune rabbit antiserum to Theiler's virus

IMMUNITY

Active Immunity

There does not appear to be any significant degree of active immunity in man on recovery from infection

Mice recovered from infection with Sanders' virus showed some resistance to an intracerebral challenge (Braley, 1945)

Serum Antibodies

Virus neutralizing antibodies develop in convalescence, and are demonstrated by cerebral inoculation in mice (Sanders, 1942, Sanders and Alexander, 1943, Sanders *et al*, 1943, Korns, Sanders, and Alexander, 1944, Braley, 1945).

Antibodies can also be demonstrated by inoculating virus-serum mixtures on the scarified rabbit cornea (Feigenbaum, Michaelson, and Kornbluth, 1945), or in the allantoic sac (Calkins and Bond, 1944).

Infection in contacts and carriers It has been estimated that about 5 per cent. of household contacts develop the disease. There seems little doubt that carriers may exist, and the sera of such apparently healthy persons may neutralize the virus of EKC (Perkins, Korns, and Westphal, 1943; Korns, Sanders, and Alexander, 1944). Doubtless such persons suffer from a subclinical attack.

Control. Various official and other sources have described the methods of control to be followed (e.g., Circular Letter No 14, 1943; Heiman, 1943, *Publ. Hlth Rep*, *Wash*, 1943). The case must be isolated, and use individual soap, towels, and washbasin. He should be instructed not to rub his eyes, and to wash his hands frequently.

Special attention should be paid to the sterilization of protective goggles or eyeshields.

Medical and nursing attendants must use scrupulous care to avoid transmitting the infection, and wash their hands at once after attending to a case.

HUMAN TRANSMISSION EXPERIMENTS

The infection can be transferred to human volunteers by conjunctival instillation of infective material from a previous case (Wright, 1930).

Sanders' virus also produces infection (Sanders, 1942; Sanders and Alexander, 1943).

THE ETIOLOGY OF EKC

Various theories have been advanced to explain the etiology of EKC, and these are reviewed by Hogan and Crawford (1942 *b*). It is now accepted that the disease is caused by a virus, although more than one agent of this group has been isolated from cases apparently typical clinically

1 A number of workers has regarded herpes febrilis virus as the cause of EKC. For example, Maumenee, Hayes, and Hartman (1945) isolated a number of strains of virus that appeared to be herpes simplex from cases of clinical EKC. The strains produced nuclear inclusions in the corneal epithelium of the rabbit They concluded that herpes virus can cause a condition identical clinically with EKC

However, herpes cornealis is a well-recognized condition (see Ch. XVII), and would not appear to resemble EKC in any significant respect Herpetic infections seldom occur in the form of epidemics, and in adults the infection is usually recurrent, the initial infection having been contracted in infancy. Hogan and Crawford (1942 *b*) do not accept the herpes virus as etiological in EKC for the following additional reasons Vesicles are very rare in EKC, and there is no ulceration of the cornea. There is no constant corneal anesthesia in EKC. The majority of workers has failed to produce a typical herpetic keratitis with consecutive encephalitis in rabbits inoculated on the cornea with material from cases of EKC In EKC the lesions usually involve both eyes

2 Dresel, Weineck, and Meding (1941) implanted tissue from the cornea on the chorio-allantois, and found that the embryo was killed One strain of agent was passed a number of times

3 Gallardo and Hardy (1943) isolated a virus ("S") from a case of cicatrizing keratoconjunctivitis, characterized by progressive cicatrization and shrinkage of the conjunctiva, more marked in the lower than upper fornices, progressive pannus, and irregular vascularization S virus closely resembled vaccinia as regards EB's, inclusions and pathogenicity for the chorio-allantois, cross neutralization tests also showed the two agents to be closely related

4 Wright (1930) claimed to have found inclusions in the corneal tissue resembling Negri or Guarnieri bodies Wright also produced changes in the rabbit cornea resembling those found in man

CHAPTER LXV

TRACHOMA

Trachoma was recognized as a disease by the ancient Greeks and Romans, being referred to by Celsus as *aspritudo* and by Dioscorides as τράχωμα (roughness).

In 1907, Halberstaedter and Prowazek discovered intracellular clumps of minute bodies in tissue scrapings derived from cases of trachoma studied in Java and, although these workers (see Halberstaedter, 1912) claimed that the structures they had found were the etiological agents of trachoma, little attention was paid to the subject at the time In 1927 interest was focused upon the claims of Noguchi (1928), who maintained that the organism he had isolated from cases of trachoma and named *B. granulosis* was the cause of the disease Subsequent investigation into the rôle of *B. granulosis* proved that Noguchi had been mistaken and that the organism was unrelated to the condition. Within the last decade studies on the morphology of psittacosis virus and the virus of inclusion conjunctivitis have renewed interest in the claims of Halberstaedter and Prowazek, since the inclusions found by them in trachoma are very similar to those occurring in psittacosis and inclusion conjunctivitis Modern work has established that trachoma is a virus disease and that the Halberstaedter-Prowazek (HP) body is the primary infective agent.

The virus bears morphological resemblance to those members of the pneumonitis-psittacosis group to be discussed in the next section, but differs from these agents in several important respects. It is most closely related to the virus of inclusion conjunctivitis, and the two agents form a biological group of their own.

The disease has always attracted much attention in the literature, and general reviews are those of Stewart (1939) and Bland (1945).

EPIDEMIOLOGY

Geographical Distribution

Trachoma occurs irregularly throughout the world. In some parts, such as Egypt, Palestine, the Levant, Algeria, Mo[illegible]
Corsica, Cyprus, certain areas of Rus[illegible]
Malaya, and Polynesia, its incidence is [illegible]
Latvia, Estonia, Poland, Lithuania, Finl[illegible]
Mexico, the Argentine, and Brazil it affects only certain sections of the population. In other countries, such as Ireland, Belgium, Holland, France, Spain, Portugal, Austria, Hungary, Germany (East Prussia), Bulgaria, Yugoslavia, Albania, Roumania, the United States, Canada and Australasia it only involves a few localities. In the United States and Canada the disease is more common in the Indian populations (Wall, 1934), Gradle (1939) estimated that there were over 30,000 cases in whites, and 25,000 in Indians in the United States The disease has been recorded [illegible]

[illegible]

cludes with the remark that "It would not be too much to say that the stigmata of the disease, active or cured, are borne by half the inhabitants of the globe. All nationalities seem to be equally susceptible to the disease, but Howard (1933 *a*) states that the Negroes in Missouri are more resistant than the whites. Scott alludes to the mild infection seen in the West African Negro (1945).

In some countries the disease is a rare affliction, being seldom seen in Britain,

No neutralizing antibodies can be detected in the first week, but after 6-10 weeks, the sera of all convalescents show antibodies, and these may persist for some months.

Rabbits can be hyperimmunized with the production of potent virus neutralizing antisera.

SPECIFIC TREATMENT

Sulfonamides and penicillin have no effect on the virus (Braley, 1945). Convalescent plasma or serum given before the 5th day of illness is the treatment of choice (Braley, 1945).

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tricial tissue forms, engulfing within its interstices the small islets of diseased conjunctival tissue, which gradually become compressed by the contracting fibrous tissue bands. Corneal pannus is evident at this stage when seen either by the naked eye or with a corneal loupe Thickening of the tarsus, trichiasis, and entropion may also develop as terminal sequelae. The clinical manifestations seen in *Tr IV* represent the final picture after the disease has run its course. Here the normal epithelium has been replaced by fibrous tissue, and likewise the subepithelial infiltration has

choma is to be found in the publications of Cange, Foley, and Parrot (1935), Cange and Foley (1924), and Julianelle and Smith, (1943)

MORBID ANATOMY AND HISTOLOGY

It is generally agreed that the principal lesion in trachoma is primary subepithelial lymphoid infiltration with lymphocytes and plasma cells, followed by degenerative

states that the primary

llicle only a secondary

of conditions prevails,

he believes that trachoma is an inflammatory and not a hyperplastic disease.

clusions upon 5 laws which have sometimes been referred to as the *laws of true trachoma*, namely: (1) that true trachoma does not exist without germinal centers or follicles; (2) the true trachoma follicle is a lymphoblasto-endothelial formation which is either confluent or follicular, (3) true trachoma develops in the most vascular part of the conjunctiva, namely the fornix cul-de-sac and the limbus, (4) true trachoma ends by degeneration, cicatrization, or hyalinization, and lastly (5) the true disease can recur after healing, but is always accompanied by the formation of fresh follicles. Pascheff, therefore, concludes that trachoma is due to hyperplasia of lymphatic structures of the conjunctiva, and is, in consequence, a lymphadenoid

processes is as yet imperfectly understood, but in the second, the formation of cicatricial connective tissue originates chiefly in the cells of the bloodvessel walls. The localization of infection in the tarsal conjunctiva expresses itself mainly in submucosal proliferation, which may develop into a massive adenoid structure with follicles

The

(1937),

tissue followed by involvement of the tarsus itself Peters (1932) has described lattice-shaped fibers which participate in the process of fibrosis, and Busacca (1933, 1934 *a*, 1935) has made a special study of Herbert's pits in trachoma. Krueckmann (1933) has drawn attention to a trachoma-like condition of the nasopharynx, and Charlin (1934) has pointed out the existence of latent trachoma The incidence of dacryocystitis in trachoma has been investigated by Aliquò-Mazzei (1937), who found it to be 4 21 per cent Milton and Beattie (1942) have also studied the histology For details regarding the biomicroscopical appearances of the limbic region, see Galante (1934), Chirkovskiy and Dymshits (1934), and Thygeson (1934 *b*).

Norway, Sweden, Denmark, Iceland, Switzerland, and New Zealand. In Britain it is a practically unknown condition, with the exception of Glasgow, and London (Sorsby, 1939); in both cities the disease is on the decline. Further information regarding the world distribution of trachoma is to be found in the *Report of the Health Organisation of the League of Nations* (1930), no. C H. 822 (1), and also in the publications of Mackenzie (1935), McMuller and Rice (1934), Tiong (1932), Busacca (1934 *b*), Cattaneo (1934), Marquez (1934), Marin-Amat and Torres (1934), Rostkowski (1934), and Stones (1939).

Susceptibility of Children and Social Factors Concerned in the Dissemination of Trachoma

Young children are very susceptible to trachoma Massoud (1935), in referring to trachoma as an endemic disease of Egypt, states that it is usually acquired in early infancy and its dissemination is fostered by errors of diet, hygiene, and other factors calculated to reduce vitality.

Bruck (1936) found that in Palestine 75 per cent. of the cases occurred in children less than 3 years old; among Fijian natives, likewise, the majority of cases developed during childhood (Stuppel, 1933), and many schoolchildren, for example, showed complete cicatrization of the conjunctiva. Sedan (1937) also alluded to the extreme readiness with which infants developed trachoma and emphasized the part played by the neglect of personal hygiene, and the evil of poverty in the spread of infection Motais (1932) quoted cases in which European children contracted the disease from their native nursemaids, while Massoud (1937) asserted that children with enlarged adenoids were highly susceptible to infection (see also François, 1935)

CLINICAL FEATURES

Onset. The condition commences with the formation of a number of rounded or irregularly shaped, solid granules of a pale yellowish-pink color, at the upper fornix. This is accompanied by clouding of the vision, especially in the mornings, but without change in the cornea. Later, translucent grayish-white round formations about the size of a millet seed develop in the deeper layers of the tarsal conjunctiva of the upper lids, and these have been regarded as a specific feature of trachomatous conjunctivitis. Occasionally there is also interference with the functioning of Muller's superior tarsal muscle, the eyelid tends to droop, and the patient's eyes present a sleepy appearance (see Falta, 1932).

The course of the disease has been arbitrarily divided by MacCallan (1936 *a*, *c*)

(3) the appearance of papillary hypertrophy, and lastly, (4) the stage of fibrous tissue formation These features contribute a basis for the classification of trachoma given below *MacCallan's classification* has been adopted universally by ophthalmologists as a sound working hypothesis for clinical description

Tr I is applied to the stage at which tiny follicles develop, or else generalized subepithelial infiltration occurs, imparting to the conjunctiva a red and velvety appearance. Rarely, these lesions may heal spontaneously, leaving behind them no trace of damage, but more freque [illegible] The second phase of the disease is characteri [illegible] cences, in which case it is called T [illegible] trophy resulting from superimposed bacterial infection, and it is designated [illegible] Conjunctival secretion is usually present at this juncture and the illness may last an indefinite period *Tr III*, which succeeds either stage *Tr I* or *Tr II*, represents the stage at which partial cicatrization or cure may occur, and a network of cica-

investigators, who also proved the contagious character of the disease. Certain cases of special interest illustrating the nature of trachoma have been reported in the publication of Cuénod and Nataf (1933), who have quoted 3 instances in which ophthalmic surgeons contracted the disease from their patients, and of Zentmayer (1934), who described the spread of infection among wrestlers in America. Other cases of interest are those recorded by Roshchin (1934) who mentions that in 11 cases of trachomatous pannus treated by him by the Dernig flap operation, the mucosa from the lip which was grafted on the surface of the conjunctiva later became invaded with trachomatous follicles.

THE RÔLE OF *B. GRANULOSIS* IN TRACHOMA

Evidence in Favor of *B. Granulosis* as the Causal Agent of Trachoma

In 1928 Noguchi recovered this organism in semisolid *Leptospira* medium from 5 cases in American Indians Colonies occurred as small grayish-white structures, the organism appeared as a motile bacillus possessing a single flagellum, and measured about 0.3 μ in its longest diameter, it was usually *gram negative*, although Trapesontzewa (1932) maintained that it varied in its reaction to Gram's stain. About 100 monkeys were used by Noguchi for inoculation experiments, and, according to Robbins (1935), several ophthalmologists who examined the animals expressed the opinion that the disease closely resembled human trachoma.

Noguchi's observation was soon confirmed by subsequent workers, all of whom were able to produce a granular form of conjunctivitis in monkeys by inoculating them with cultures of *B granulosis* isolated from cases of trachoma, and, among others, the following have recorded their results in the literature Tyler (1929), Olitsky, Knutti, and Tyler (1931), Reimann and Pillat (1931), Wilson (1931), Finnoff and Thygeson (1931), Bengtson (1932 *b*), Proctor, Finnoff, and Thygeson (1932), Thygeson (1932), Tallo (1932), Weiss (1933 *b*), Olitsky (1934).

These researches were repeated by other workers. Thus, in the *United States* it was observed by Tilden and Tyler (1930), Kendall and Gifford (1930), Olitsky, Knutti, and Tyler (1931), Finnoff and Thygeson (1931), Thygeson (1932), and Weiss (1933 *c*) In *Italy* by Bordonaro (1929), Addario (1930), Bietti (1930), and Tallo (1932) In *Tunis* by Weiss (1933 *c*) and Thygeson (1933 *a*) In *Egypt* by Olitsky, Knutti, and Tyler (1931). In *Russia* by Berberov and Alekseev (1932) and Trapesontzewa (1936) In *Hungary* by Kanyó and Rotth (1932). In *China* by Reimann and Pillat (1931) In *Japan* by Takamatsu (1932). In *Scotland* by Meighan and Urquhart (1936).

Certain observers considered that conjunctivitis reproduced in monkeys resembled the lesions of human trachoma, but Thygeson (1932, 1933 *b*), for instance, pointed out that the two were not identical, since cicatrization and pannus were absent in the monkey Furthermore, another objection was raised on the ground that monkeys sometimes suffered from spontaneous follicular conjunctivitis, but, according to Weiss (1933 *a*), this condition was different from that produced after experimental inoculation with *B granulosis* Some writers suggested that inability to reproduce typical trachomatous lesions in monkeys might have been due either to natural resistance or to loss in virulence of the cultures (see Meighan and Urquhart, 1936)

Failures to Isolate *B. granulosis* from Cases of Trachoma

Among other workers, the following have reported that they were unable to find *B granulosis* in cases of trachoma investigated by them in different parts of the world the *United States* (Bengtson, 1932 *a*), *Tunis* (Thygeson, 1931), *Egypt* (Wilson, 1931), *Roumania* (Puscariu and Nitzulescu, 1932), *Poland* (Lawrynowicz and Melanowski, 1930), *China* (Tang, 1930, 1934), *Germany* (Rohrschneider and

Cytology of the Conjunctival Secretion

This has been studied by Marcus and Wainer (1936), who point out that the results of such examination assist in the differential diagnosis of trachoma from follicular conjunctivitis. Thus, in the former it is common to find embryonal forms of lymphocytes with vacuolated protoplasm and nuclear mitoses, the so-called "shadow cells" of Humprecht resulting from cytolysis of these elements, and epithelial cells exhibiting degenerative changes of their nuclei. In follicular conjunctivitis, however, embryonal cells are absent, histiocytes are rare, the epithelial cells are normal in their appearance, and eosinophils are commonly present.

CLINICAL PATHOLOGY

The *lysozyme content* in normal tears has been compared with that found in the lacrimal secretion derived from 64 cases of trachoma by Mourzinn and Souchkowa (1935). These workers showed that the titer was considerably diminished during the acute stages of trachoma and particularly so in those cases in which corneal ulceration existed. There was a tendency for the lysozyme content gradually to return to normal as healing occurred.

The *blood picture* in trachoma has been investigated by François (1931), who found that in chronic cases a neutropenia existed accompanied by a relative lymphocytosis and monocytosis. Mihail (1936) made a similar reference to the blood picture and observed that monocytes were more numerous than lymphocytes, and that no eosinophilia occurred without concomitant parasitic infection.

INCUBATION PERIOD OF TRACHOMA

In the case of the naturally acquired disease the incubation period cannot always be estimated accurately. MacCallan (1936 *c*) points out that the onset of the disease is usually insidious and symptomless, so that the exact period is diffiult to assess.

On the other hand, in the experimentally induced disease the incubation period is comparatively brief, and from the results of tests performed on human volunteers it was found that
and Vancea (1932)
days by Delanoe (
mental trachoma is usually an acute illness from the start, characterized by a short incubation period, whereas the natural disease is slow in its onset and chronic from the beginning (see Michail and Vancea, 1932; and Howard, 1933 *a*) This fact was appreciated as long ago as 1900, and Halberstaedter (1912) mentions that, when Addario first announced that the incubation period of the experimental disease in man was 3 to 6 days, Greeff, Frosch, and Clausen (1908) repeated the work because Addario's results were contrary to the opinion current at the time (see also Addario, 1910).

To-day these facts are well established, and the incubation period of experimentally induced trachoma is accepted as being of shorter duration than the naturally acquired condition At the present time there is a growing tendency among a section of writers (see Foley and Parrot, 1937 *a*) to regard the virus of trachoma as one possessing feebly invasive properties If this assumption is proved to be correct, it may be argued that the clinical differences existing between naturally and artificially acquired trachoma are explicable on the grounds that a greater quantity of virus is implanted into a susceptible conjunctiva by direct injection than is possible in natural contagion

INFECTIVITY IN MAN

Addario in 1900 was the first to reproduce the disease in human volunteers by inoculating the conjunctivae with tissue scrapings derived from the eyelids of trachomatous subjects, and this work was confirmed by Greeff (1909) and other

The preparation of films.

The secret of success in preparing films lies in the art of removing as many epithelial cells as possible from the conjunctival surface In order to accomplish this, the eyelid should be everted, freed from pus by washing it with salt solution, and the surface scraped with a single stroke of a small knife, taking care to cause as little bleeding as possible. The epithelial scrapings are now placed on a clean glass slide and spread evenly over its surface with the aid of another glass slide, after the usual manner of preparing a blood film, exerting only the minimum amount of pressure when so doing. Films prepared by rubbing a platinum loopful of conjunctival secretion on the surface of a slide must not be used when attempting to demonstrate inclusion bodies, likewise cotton wool swabs are unsatisfactory for this purpose.

Staining by Giemsa's method.

Fixation. Preparations are dried in air, treated with either methyl alcohol, absolute alcohol, or a mixture of equal parts of absolute alcohol and ether for 10 minutes and then dried with blotting paper.

Staining. A freshly prepared solution of Giemsa's stain is required

12 parts Giemsa's eosin solution, consisting of 2.5 c c. of 1 per cent. French eosin solution to 500 c c of distilled water.
3 parts azure I (diluted 1 in 1,000).
3 parts azure II (diluted 0 8 in 1,000)

Procedure The fixed preparations are immersed for 6 hours in the solution, removed at the end of that time, rinsed with water and dried with blotting paper The method has given excellent results and was originally recommended by Halberstaedter and Prowazek (1907 *a*, *b*, 1909, 1910), and Greeff, Frosch, and Clausen (1908) Various modifications of this method have been used with equal success by other workers

Greeff (1909) stained for from 6 to 9 hours at a temperature of 37° C, or with the same mixture for 3 hours at 56° C.

Herzog (1909, 1910) employed a 1 in 40 dilution of commercial Giemsa's solution in distilled water and stained the films with it for 1½ hours at 37° C

Heymann (1909, 1910) preferred to use a 1 in 20 dilution of the same stain applied for ¾ hour at room temperature, and Bertarelli and Cecchetto (1909) employed a 1 in 11 dilution for 24 hours

Lindner (1910 *a*) advocated the employment of 2 methods which accentuated the basophilic nature of the inclusion bodies, films were dried in air and fixed with absolute alco[illegible]
procedures [illegible]
of 10 c c. of [illegible]
acetic acid f[illegible]
solute alcohol, and examined under the microscope. Method B consisted of staining the slide for 10 minutes in a solution consisting of 10 drops of Giemsa's stain mixed with 10 drops of 0 5 per cent acetic acid, after which it was washed with distilled water, dried in air, mounted in cedar-wood oil, and examined as usual In preparations stained by these methods, the inclusion bodies, the granules of mast cells, and contaminating bacteria stained blue, the nuclei of lymphocytes and leukocytes also [illegible] than the [illegible] intensely

Sections can also be stained in this way Tissue is fixed in sublimate or formol Sections are stained for 12 to 48 hours in the solution employed in method A, immersed in distilled water for ½ to 1 hour, dehydrated with absolute alcohol and xylol, and mounted in cedar-wood oil. An alternative procedure is to stain with

Heymann, 1931), *France* (Lumbroso and van Sant, 1931; Morax, 1930); and *England* (McCartney and Mayou, 1930).

Negative Results following Experimental Inoculation of Man

Cultures of *B. granulosis* have been injected into the conjunctivae of blind persons, but we are unable to quote a single instance from the literature in which typical trachoma was reproduced by this means. In a few cases a transient and mild inflammatory type of conjunctivitis ensued following inoculation of the organism, but, apart from such a response (which is only to be expected if living bacteria are injected into healthy tissues), true trachomatous cicatrization and pannus have never been produced by *B. granulosis* (see Proctor, Finnoff, and Thygeson, 1932, Thygeson, 1933 *a*, *b*, Candian, 1933; Lindner, 1929; Howard, 1933 *b*). Berberov and Alekseev (1932) inoculated the eyes of children but the organism failed to reproduce the disease, thus conclusively proving that *B. granulosis* is not the cause of human trachoma (see also Thygeson, 1932, 1933 *a*, *b*, Wilson, 1931; Robbins, 1935).

The Rôle of *B. granulosis* Investigated Serologically

[illegible] to Weiss (1933 *c*), the sera of [illegible] *granulosis* more frequently tha[illegible]ment fixation reactions, in which the organism was used as antigen against the patient's serum, have also been described by Trapesontzewa (1937). On the other hand, Fischer-Ascher (1931) failed to secure either positive complement fixation or agglutination reactions in his experiments, and Trapesontzewa (1932) reported negative agglutination tests between the organism and human serum Mikaelian (1931) likewise obtained negative skin tests after injecting emulsions of *B. granulosis* intradermally.

Conclusions

The divergence of opinion suggests that *B. granulosis* is not the primary etiological agent, but is more probably a secondary invader Investigations by Harrison and Julianelle (1936) revealed that the bacterial flora of the eye in trachoma was in no way different from that of the normal conjunctiva Under the circumstances, it must be concluded that, so far, it has not been possible to isolate any specific organism from cases of human trachoma, or to reproduce the disease in man by the injection of *B. granulosis* or any other bacterium.

THE INCLUSION BODIES OF TRACHOMA

It is generally agreed that the Halberstaedter-Prowazek inclusion bodies found within the cytoplasm of the superficial epithelial cells of the conjunctiva are most numerous during the acute stages of trachoma For example, Rotth (1932) stated that they were noticed by him in practically every case during the early stages of the illness, but as the condition progressed they became less frequent, and in patients who had suffered for over 5 years, they were found in only 50 per cent of persons examined

MacCallan (1936 *b*) also alluded to the same point, mentioning that inclusion bodies were commonly found in the first stage (*Tr I*) of trachoma and that in the later phases of the condition they became scanty in stained films In Japan Onisi (1936) found that inclusions were most numerous in patients who lived in the south of the country.

Staining of the Halberstaedter-Prowazek Inclusions of Trachoma

Halberstaedter's (1912) classical account of the methods employed for staining these inclusion bodies, contained in Prowazek's *Handbuch der Pathogenen Protozoen*, is as yet unsurpassed

dumb-bell. Pear-shaped and hourglass-like forms have also been observed, and these are best demonstrated in wet fixed films which have been treated with Heidenhain's iron hematoxylin stain (Leber and Hartmann, 1909).

Large inclusion bodies.

These usually appear in the cytoplasm of infected epithelial cells, but according to Leber and Hartmann have been detected in the nucleus.

Clumps of elementary bodies.

These are often found in the cytoplasm of epithelial cells, and may be small in size or else they may form a large compact mass lying free in the cytoplasm or clinging to the surface of the nucleus Alternatively, the elementary bodies may be so numerous as to fill completely the cytoplasm of the cell and, less commonly, they may appear evenly distributed throughout the cell, each elementary body being embedded in a pale blue-staining mass of protoplasm referred to by Halberstaedter (1912) as "plastin-like"[1] material, to which we shall make repeated references in the text. The following is a translation of Halberstaedter and Prowazek's classical account of the inclusion bodies of trachoma studied in Java:

"In the Giemsa preparations, dark blue-stained nonhomogeneous irregular inclusions were visible beside the nucleus in the pale blue protoplasm of the epithelial cells. The inclusions were at first small, round or oval in shape but they gradually grew in size and finally assumed the appearance of a mulberry During this process the inclusion, commencing from its center, became less dense in character. Gradually the inclusions tended to clasp the nucleus like a cap and later fine red-stained corpuscles appeared within these inclusions, and as they rapidly multiplied the blue masses disappeared simultaneously. Finally the granules or corpuscles (*elementary bodies*) occupied the greater part of the protoplasm and then the blue-stained substance only became visible as small islands between the mass of granules. In smear preparations, free granules were also found extracellularly.

"The changes described in human conjunctival epithelium could be transferred from man to the orang-utan and also from one ape to another by experimental inoculation. The blue amorphous masses of the inclusions had the same affinity for the blue component of the Giemsa dye as the nucleoli and were probably identical with plastin The circumscribed granules, on the contrary, which were distinctly red and approximately 0 25 μ in size, probably represented the virus itself, for they multiplied rapidly by growing and then dividing into two colon-like granules"

The initial bodies of Lindner.

These bodies have been described in great detail by Lindner (1909 *a*, *b*, 1910 *a*, *b*), subjected to repeated investigation by later workers, and have been observed not only in the cytoplasm of epithelial cells in trachoma, but also in inclusion conjunctivitis of the newborn child (see p 718). Lindner and others have regarded them as the initial forms of the virus from which the elementary bodies are developed

and multiplies by repeated division to form groups and clusters of initial bodies which become embedded therein As growth proceeds, minute red-staining elementary bodies commence to appear inside the initial bodies, this process starts in those occupying the middle of the inclusion body mass, so that, after a time, the center of the inclusion cavity becomes filled with elementary bodies, and only a few initial bodies remain at the periphery of the cell Later, elementary bodies begin

[1] The reason for adoption of the name "plastin" does not appear to be very clear to us, but we have given prominence to it in our account since it indicates that those who observed its presence recognized that, following the entry of a virus, the cell was liable to react against the invader

a solution of 2 drops of Giemsa stain added to 15 c.c. of distilled water for 2 to 3 days, and then dehydrate the preparation with acetone, acetone-xylol, and xylol

Flemming (1910) first treated the film with old Giemsa solution for 5 hours, then carried out differentiation with a solution of absolute alcohol and 1 per cent. acetic acid, watching the preparation continuously under the microscope until nothing but the cell nuclei and trachoma inclusions remained visible. The former appeared a reddish-violet hue and the latter blue or red in color, depending on the stage of their development. For other methods of staining the inclusion of trachoma, see Bertarelli and Cecchetto (1909), and Botteri (1912).

Staining by Heidenhain's hematoxylin.

Heidenhain's iron hematoxylin stain has been highly recommended by Leber and Hartmann (1909).

Fixation Wet film preparations are treated with sublimate, alcohol, or Hermann's fluid.

Staining. Heidenhain's hematoxylin stain is applied for 24 hours.

The appearances of the elementary bodies when stained by this method are described by Leber and Hartmann as being deep black in color; the cytoplasm of the cell is light gray. The same method was employed by Herzog (1910) and Radziejewski (1909), both of whom recommended it, the former advocating its suitability for staining sections

Method of demonstrating inclusion bodies in sections.

Embedding of tissue.

1. Excise small fragments of tissue from the conjunctiva.
2. Fix in 3 per cent. sublimate alcohol containing 3 per cent. acetic acid
3. Heat to 50° C. for 1 hour.
4. Rinse in water.
5. Treat with dilute alcohol, absolute alcohol, and finally iodized alcohol for 1 hour.
6. Remove iodine with absolute alcohol
7. Treat with aniline oil, and fresh xylol several times.
8. Embed in soft paraffin for 1½ hours, and next in hard paraffin
9. Cut sections less than 5 μ in thickness, and mount on glass slides

Staining.

1. Dilute Weigert's hematoxylin solution to half strength and apply for 2 to 3 days.
2. Differentiate the slide in 1.5 to 4 per cent. iron alum solution, or in 30 per cent acetic acid, until the chromatin of the epithelial cell nucleus retains a distinct hematoxylin color, wash, dry, mount, and examine as usual

Morphology of Inclusion Bodies

Single granules.

In films stained by Giemsa's method (described above) prepared from conjunctival secretion in an acute case of trachoma, discrete extra- and intra-cellular granules can be detected These were referred to as "chlamydozoa of trachoma" by Prowazek (1907). They stain reddish in color and were subsequently renamed "elementary bodies" or "elementary organisms" by Herzog (1909), and are now recognized as being identical in morphology with the elementary bodies found in other virus diseases In size these minute particles measure 0.25 μ in diameter, and are surrounded by a halo of nonstaining material (the significance of which will be discussed later), they are frequently present in pairs, and are sometimes connected by a slender filament so that the whole structure resembles the outline of a

being present In sections, encapsulated bodies are found in the superficial epithelium, but not in the deeper layers. He also found granular inclusions in recticulum cells in the subepithelial layers

The Interpretation of the Trachoma Inclusions

The histological appearances presented in stained films and sections described above are difficult to explain owing to the number of possibilities which have to be considered Thus, it is conceivable that all the different types of inclusion body mentioned may represent a life cycle of changes exhibited by the parasite during the course of its development. On the other hand, it is possible that some of the inclusions may be forms of the living parasite, and others the reaction of the invaded cell towards it. A combination of these 2 factors is also likely to account for the so-called intermediate varieties mentioned. In discussing this problem Halberstaedter (1912) has suggested that the complete inclusion body might be constructed of 3 entirely different parts, the first of these is the blue-staining so-called "plastin-like" substance which Halberstaedter believes is a derivative of the cell cytoplasm, the second the red-staining parasite which appears as a clump of granules, and the third, a structureless matrix in which the elementary bodies are embedded The last-named component is of particular interest because it suggests that the elementary bodies do not lie free inside the inclusion body cavity, but are embedded in a gelatinous medium. Halberstaedter believes that it is the presence of this substance surrounding the elementary body which gives rise to the clear halo of nonstaining material sometimes evident in Giemsa-stained films He con-

scribed as plastin, on the contrary, is deposited on the elementary bodies which it covers like a shell, and its amount may either be plentiful, in which case it appears as coarse clumps, or else it may be sparsely distributed According to the same authority, the plastin substance not only represents the "defensive mechanism" of the host cell, but is also used by the elementary bodies as a nutritive medium,

Halberstaedter's conception of the composition of inclusion bodies of trachoma, based on a study of stained films, is of considerable interest to the authors, since,

of trachoma

The authors have observed the halo or nonstaining zone which sometimes surrounds individual elementary bodies in Giemsa-stained film preparations, and are in complete agreement with Halberstaedter's opinion that this clear area is formed

r their libera-

(1937 a), and

Foley and Parrot (1937 a) supports the contention that the inclusion bodies of trachoma represent a living virus and are not inanimate cell products The possibility of trachoma being an infection due to the symbiotic combination of a virus with a bacterium has been discussed by Thygeson (1934 c) and Pittaluga (1934) The following are some of the other opinions expressed on the trachoma inclusion

to develop inside the marginal initial bodies, until eventually all of these disappear and the cell becomes packed with elementary bodies.

Initial bodies have been observed by Lindner to occur not only in film preparations, but also in stained histological sections, and the following different shapes have been recognized. *ring forms* appearing as little *disks* stained at their edge, *diplococcus-like varieties* exhibiting polar staining, *oblong types*, being either rod-shaped in outline or else possessing a lateral curvature, and also *sickle-shaped forms*, all of which have been seen to occur not only as intracellular inclusions but also as free extra-cellular bodies. Thus, *free-lying initial bodies* have been described by Mijaschita (1908), Botteri (1912), and Thygeson (1934 *a*), but Stewart (1934), failed to find them in 300 cases examined in Egypt.

The residual bodies (*Restkörpter*) of Halberstaedter and Prowazek

These are found inside the cell inclusion alone, and stain red with Giemsa, or blue by Mallory's method In size residual bodies seem to be larger than the initial bodies, but as the elementary bodies become more numerous in the cell, they diminish in size correspondingly. Lindner believed that the residual body was not a growth phase of the virus manifested during the course of its development, but a nuclear fragment, basing this statement on the fact that since the residual body only occurred at one part of the inclusion body and could be stained with methyl green, it was unlikely that it was a living organism.

Plastin-like masses.

Amorphous masses appearing within the cytoplasm of parasitized cells have been referred to by Halberstaedter (1912) as plastin-like masses They are stained blue with Giemsa's solution and, since they show the same affinity for this dye as do the nucleoli of cells, it has been suggested that they are degenerative products (e g, by karyorrhexis) of the cell and not associated with the parasite.

Intermediate varieties of inclusion body.

Although the various forms of inclusion body already mentioned have been described as sharply defined structures, it is obvious from a survey of the literature that a multitude of intermediate varieties, which cannot be placed in any distinct category, exist. Whether or not these represent distinct phases of the parasite itself, or are made up of a combination of some of the forms described, it is impossible to tell, but Herzog (1909, 1910) has described one variety of inclusion body which can be assigned to the latter class This cytoplasmic inclusion consists of 2 components, the one a clump of red-staining elementary bodies and the other a thin blue envelope which surrounds them, so that the pink elementary bodies shining through the blue covering impart a purple tinge to the whole structure, and it was to describe this outer covering or "mantle" (χλαμύς) that Prowazek originally coined the term *Chlamydozoa*

Microchemical Properties of the Inclusions of Trachoma

Rice (1936) has made a series of interesting observations on the color reactions exhibited by the Halberstaedter-Prowazek inclusion body after treatment with iodine and other chemical reagents, from which he concluded that its outer surface is composed of carbohydrate material (see also Thygeson, 1938 *a*, Thygeson and Richards, 1938)

Later Observations

A number of later workers has studied HP inclusions (Braley, 1940, Julianelle, 1940 *a*, *b*; Loewenstein, 1945) Julianelle (1940 *b*) reports that tissues with inclusions are twice as infective for monkeys as tissues without Loewenstein (1945) reports that in scrapings the inclusions are polymorphic, individual EB's and inclusions

in the literature that occasionally volunteers who had been experimentally inoculated with infective material sometimes failed to develop the disease. This would appear to suggest either that such persons were immune to trachoma or else that, before the virus was able to establish itself in the healthy conjunctiva, a certain amount

the filtrate it usually proves to be nonpathogenic (see Thygeson and Proctor, 1935).

Experiments using Collodion Membranes

In 1934, Thygeson concluded that the Halberstaedter-Prowazek inclusion bodies and elementary bodies of trachoma represented intracellular colonies of a virus in different stages of its development. He moreover showed that these bodies could be concentrated by high-speed centrifugalization, and were small enough to traverse the pores of a collodion membrane filter possessing an average pore diameter of less than 0.2 μ.

Later, *Thygeson and Proctor (1935) further investigated this problem, and* again proved that the virus of trachoma was readily filterable through collodion membranes. In their experiments fresh trachomatous tissue was removed from 14 cases occurring in American Indians, suspended in broth of pH 7.6, clarified by passage through filter paper, and then passed through a collodion membrane 0.3 mm thick (possessing an APD of 0.75 μ), at a negative pressure of 150 mm. of mercury applied for 5 minutes Filtrates obtained by this method were free from bacteria, and 2 weeks after inoculation into the conjunctivae of 5 African sphinx baboons, typical trachomatous follicles developed at the site of injection Similar lesions were produced in baboons inoculated with unfiltered material.

Preservation

The virus dies rapidly outside the human body and, as yet, no method has been devised by which the infectivity of material can be preserved for any lenth of time.

There has been some divergence of opinion with regard to the exact length of time that the virus survives outside the body, but data based partly on human test inoculations and partly on animal results, as a whole suggest that the virus is readily destroyed. Table 30 shows a summary of findings obtained by different workers Julianelle and Harrison (1938) were able to preserve the infectivity of material for 7 days by suspending the tissue in veal infusion broth of pH 7.6 followed by storing it in an ice-chest at 4° C. In 50 per cent glycerol it remained active for 2 weeks, and Julianelle and Harrison (1938) showed that it was able to resist alternate freezing and thawing 16 times.

Trituration

Mechanical grinding and emulsification of trachomatous tissue in saline have no effect on the viability of the virus, according to Stewart (1933, 1934), and Julianelle and Harrison (1938).

Desiccation

According to Hess and Romer (1906), dried material lost its infectivity after 4 weeks, and Botteri (1912) found that material desiccated for 1½ hours at 15° C. lost its infectivity for baboons Nicolle, Cuénod, and Blaizot showed that drying for 30 minutes at 32° C. inactivated the virus Likewise, Julianelle and Harrison proved that infective material suffered considerable loss of pathogenicity when maintained in the desiccated state for a few days.

Flemming (1910), for example, denies the existence of any developmental forms of the parasite, and distinguishes 3 separate types of inclusion, namely, Type I, corresponding to the initial forms, Type II, the small cap-like inclusions with plastin still adherent to them, and lastly, Type III or fully developed bonnet-like inclusions composed of elementary bodies. Gruter (1938) does not think that these structures are virus bodies, but maintains that the Halberstaedter-Prowazek inclusions are simple spherical Golgi bodies which have increased in size through repeated cell division under the stimulus of inflammation, further, during the course of the trachomatous process he has not detected any structures simulating parasitic organisms. Stewart (1934) states that "the Halberstaedter-Prowazek inclusion body is a phagocytic vacuole in an epithelial cell. It may contain any microorganism which is present in the conjunctiva either bacterial or granular." In the same paper Stewart severely criticizes earlier work on the subject. Thygeson (1934 *a*), on the other hand, has contradicted the findings of Stewart, and asserts that the inclusions of trachoma are not phagocytosed bacteria, but typical intracellular forms of a filterable virus which exhibits different stages in its development

PROPERTIES OF THE TRACHOMA VIRUS

Filtrability

Attempts to show that the infective agent of trachoma belongs to the group of so-called filter-passing organisms have in the majority of experiments yielded negative results For the convenience of the reader, we have summarized the results of different workers in Table 29, and from these it will be observed that, while a few persons have been successful, many have failed to show that the virus is capable of traversing
nonpathogenicity of
the principal one bei
Certain workers have maintained that the virus is one which is only capable of producing a low-grade localized infection of the conjunctiva and is unable to invade other tissue. According to MacCallan, all races of human beings are equally susceptible to infection, but it is quite possible that certain individuals may possess a greater degree of resistance to infection than others, for example, we have noticed

TABLE 29

Summary of Filtration Experiments

Type of Filter	*Source of Material*	*Fluid Used for Suspension*	*Filtrate Inoculated in*	*Result*	*Authority*
Berkefeld	Man	Saline	Man	Negative	Pfeiffer and Kuhnt (1905)
Berkefeld	Man	Saline	Man	Negative	Hess and Romer (1906)
Berkefeld	Man	Saline	Man	Negative	Candian (1933)
Berkefeld V	Man	Saline	Man	Negative	Fermi and Repetto (1907)
Berkefeld V	Man	Saline	*M sylvanus*	Positive	Nicolle, Cuénod, and Blaizot (1913)
Berkefeld V	Man	Tears	Chimpanzee	Positive	Nicolle, Cuénod, and Blaizot (1913)
Berkefeld V	Man	Saline	*M rhesus*	Negative	Julianelle and Harrison (1933)
Berkefeld V	Man	Broth of pH 7 8	Baboon	Negative	Stewart (1933)
Berkefeld N	Man	Broth of pH 7 5	Man	Negative	Lumbroso and Thygeson (1933)
Berkefeld W	*M cynomolgus*	Saline	*M cynomolgus*	Positive	Bertarelli and Cecchetto (1908)
Berkefeld W	*M rhesus*	Tyrode	*M rhesus*	Negative	Weiss (1933 *c*)
Pasteur-Chamberland L_1 and L_2 grades	Man	Rabbit serum 10 per cent	Man	Negative	Lumbroso and Thygeson (1933)

reported (Harrison and Julianelle, 1937, Julianelle, Harrison, and Morris, 1937). Santoni (1940) grew human corneal cells, but could not demonstrate invasion by the virus.

Various workers have attempted to adapt the virus to the chorioallantois or yolk sac, but no definitely positive results have been obtained (Wright, 1937; Vancea, 1940, Burnet, Cuénod and Nataf, 1941, Julianelle, 1943).

Animal Inoculation Experiments

Before reviewing the reports of various workers on this subject the reader is reminded that in every experiment in which unfiltered human conjunctival material has been directly inoculated in the conjunctivae of apes or monkeys, the results are open to certain obvious criticisms This is so because neither normal human conjunctival secretion nor that of lower animals is free from bacteria, and pathological effects due to the presence of bacteria must be taken into consideration when assessing the significance of experimental results. Thus, for example, in the case of a monkey which has been injected with human conjunctival material, it is difficult to tell with certainty whether or not the lesions produced in the latter have been caused by the virus of trachoma or by a secondary invading bacterium The possibility of spontaneous conjunctivitis being developed by the animal resulting from traumatization of the mucous membrane is another chance that must be remembered (see Lumbroso, [illegible]) So far, no worker has [illegible]
in monkeys, apes, or ot [illegible]
those of human trachor [illegible]
doubt, however, that [illegible]
it has been appreciated by many investigators that all such experiments should be carefully controlled and that the utmost caution is required when interpreting the results The necessity for the adoption of such a critical attitude will be appreciated when it is recalled to memory that many of the experienced ophthalmologists who saw monkeys which had been inoculated with cultures of *B. granulosis* by Noguchi were deceived into believing that the animals were suffering from trachoma (see Robbins, 1935)

The susceptibility of apes and monkeys to trachoma.

In the experimental animal, trachoma is a mild affection which is not followed by cicatricial changes or pannus.

Hess and Romer (1906) were the first investigators to reproduce a disease resembling trachoma in the baboon by experimental inoculation with human material, this taking the form of a conjunctivitis which healed completely without scar formation Bajardi (1907) produced slight lesions in the macacus and cercopithecus monkeys, Bertarelli and Cecchetto (1908) were able to infect a variety of lower ape of the species *Inuus cynomolgus* after an incubation period of 17 days, Lindner likewise obtained a positive result using a lower ape, Kuht and Thielmann (according to Halberstaedter, 1912) were successful with Java apes, and Weiss (1933 *c*) employed the New World monkey *Cebus capucinus* Halberstaedter and Prowazek (1907 *a*, 1909) were able to infect the orang-utan, and transmit the [illegible]

[illegible] Thygeson and Proctor (1935) transmitted trachoma to 5 African sphinx baboons (*Mandrillus sphinx*) by inoculating them with bacteria-free filtrates prepared from human material (see also Lumbroso and Thygeson, 1933)

The *Macacus rhesus* monkey has been employed by Julianelle and Harrison (1933), but McCartney and Mayou (1930) and Stewart (1934) found that these

TABLE 30

EFFECT OF TEMPERATURE ON TRACHOMA VIRUS

Treatment of Material	*Fluid Used for Suspending Tissue*	*Effect on Virus*	*Authority*
15 minutes at 37° C	Normal serum	Nil	Julianelle and Harrison (1938)
90 minutes at 37° C.	Saline	Nil	Mijaschita (1908)
3 to 4 hours "at room temperature"	Tyrode of pH 7 4	Nil	Stewart (1933)
3 to 4 hours "at room temperature"	Broth of pH 7 4 to 7 8	Nil	Stewart (1933)
3 to 4 hours at 20° C	Patient's blood	*Nil*	*Julianelle and Harrison (1938)*
6 to 48 hours at 5–6° C	Dog plasma	Nil	Baroni and Michail (1932)
24 hours at 0° C.	Material frozen in fresh state	Nil	Julianelle and Harrison (1938)
24 hours at 37° C.	Dog plasma	Nil	Baroni and Michail (1932)
24 hours at 35–37° C	Normal human serum Tyrode of pH 7 4	Nil	Stewart (1933)
3 days "at room temperature"	None	Nil	Baroni and Michail (1932)
7 days at 40° C.	Veal infusion broth of pH 7 6	Nil	Julianelle and Harrison (1938)
2 minutes at 120° F	Saline	Killed	Wright (1937)
15 minutes at 45–50° C	Veal infusion broth	Killed	Julianelle and Harrison (1938)
30 minutes at 50° C	Saline	Killed	Nicolle, Cuénod, and Blaizot (1913)
30 minutes at 58–63° C	Saline	Killed	Hess and Römer (1906)
45 minutes at 0° C or 10° C	Saline	Killed	Botteri (1912)
90 minutes at 35° C	Distilled water or Tyrode of pH 6 4	Killed	Stewart (1933)
3 hours at 43° C.	Saline	Killed	Botteri (1912)
4 hours at 15° C	Saline	Killed	Botteri (1912)
12 to 48 hours (tested *in vivo*)	In anterior chamber of rabbit's eye	Killed	Baroni and Michail (1932)
24 hours at 37° C	Tyrode, saline, or veal infusion broth of pH 7 6	Killed	Julianelle and Harrison (1938)
24 hours at 0° C	50 per cent glycerol	Killed	Stewart (1933)
2 to 3 days at 0° C	Broth or saline	Killed	Julianelle, Harrison, and Morris (1937)
6 to 10 days at 37° C	Dog plasma	Killed	Baroni and Michail (1932)
10 days at 37° C	Saline	Killed	Mijaschita (1908)

Centrifugalization

Low speed centrifugation for a short period of time had no effect in depriving the supernatant fluid of its infectivity (e g 1,500 r p m. for 5 minutes), but 30 minutes at 5,000 r p m was sufficient to render the supernatant fluid noninfective for monkeys (Julianelle and Harrison, 1938). The agent appears to be intimately linked with the tissues of the conjunctiva, and the washing of fragments by repeated centrifugalization and successive changes in saline fails to deprive them of their infectivity, according to Stewart (1933).

The Action of Certain Organic and Inorganic Compounds on the Virus

Trachoma virus is destroyed in 15 minutes at 37° C by the addition of a 25 to 35 per cent. solution of bile It is also inactivated after 3 to 4 hours by each of the following strengths of fluids 1 in 100,000 gentian violet, 1 in 1,000 tartar emetic, 2 per cent silver nitrate, 0 25 per cent phenol, and 4 per cent. cocaine (Julianelle and Harrison, 1938)

Cultivation

Attempts have been made to cultivate the virus, using the standard methods of Maitland and Maitland (1928), Li and Rivers (1930), Woodruff and Goodpasture (1931), and Burnet and Galloway (1934) No definite growth has been

been negative, according to Weiss (1933 c) Krümmel (1937) claims to have transmitted the infection from the human being to the conjunctiva of the rabbit.

Guinea-pigs The earlier experiments performed on these animals were uniformly negative Recently, von Szily (1935) reports that 2 to 3 months after intracerebral inoculation of guinea-pigs with trachomatous tissue, small nodules about the size of a millet seed appeared in the meninges Histological examination of these lesions failed to disclose any evidence of cellular reaction, and no perivascular infiltration or cuffing could be seen. The follicles in the meninges were comparatively large in size and occurred in the dura mater as well as in the pia and between the two membranes. They were distributed over a wide area and appeared to lie at some distance away from the blood vessels. The intra-ocular route of inoculation was also employed

Mice Attempts to infect these animals have failed, and Weiss (1933 c) reported that efforts to adapt the virus to mice by repeated intracerebral inoculation and passage were unsuccessful.

Goats and *dogs* have been inoculated, but without success, although in the past Schiele (1910) claimed to have obtained one positive result in a dog (see also Greeff, Frosch, and Clausen, 1908).

IMMUNITY IN TRACHOMA

Since trachoma is liable to recur in patients who have previously suffered from the condition, Julianelle and Harrison (1938) have studied the question of immunity to the disease in experimentally infected monkeys, and found that animals which had recovered from one attack of trachoma were susceptible to a second It was also found that the sera of human convalescents or normal persons failed to neutralize the virus when tested by inoculation of monkeys For these experiments conjunctival scrapings were suspended in broth, mixed with serum, whole blood, or plasma, kept at room temperature for 3 to 4 hours or 12 hours, or sometimes at 37° C for one hour only, and the mixture inoculated in monkeys No neutralization was, however, demonstrable by any of these procedures, and the absence of antibodies to the virus questions the value of serum therapy in the treatment of human trachoma. Julianelle (1939) has confirmed the low antigenic potency of the virus in monkeys

The intradermal test (Tricoire, 1931).

The antigen used for injection was prepared as follows Trachomatous granulation tissue was scraped from the surface of the conjunctiva and to it was added 10 times its volume of physiological saline The material was thoroughly ground up with a pestle in a mortar, then stored at 37° C. for 2 weeks, at the end of this time enough tincture of iodine was added to yield a final concentration of 1 per cent The dose injected consisted of 0,25 c c introduced intradermally with a fine needle after the usual manner of performing a skin test A positive result was signified by the formation of a red papule which developed in 48 hours and remained visible for 2 days In a negative test no reaction occurred, as the fluid was absorbed without any visible change on the skin surface

Practical value of the test Sedan (1933) performed the test on 150 persons suffering from the florid variety of trachoma, and found that 61 3 per cent gave a positive response, 16 per cent a doubtful result, and 22 6 per cent a negative reaction Control experiments were also performed on nontrachomatous subjects, and in these individuals the reaction was negative in the majority of instan[illegible] in earlier paper, however, Sedan (1931) reported that cases of tuber[illegible] syphilis sometimes gave a positive Tricoire test. Belot (1931) also inves[illegible] practical utility of the reaction in a series of 511 cases under his care and at the conclusion that the test was unreliable In his series of 400 cases of

monkeys were relatively resistant to infection. Blanc, Pages, and Martin (1939) used *M. sylvanus*, and after passage were able to infect man.

The susceptibility of different species of ape and monkey to trachoma virus has been studied by Stewart (1934), who found that the species most susceptible to experimental infection were monkeys of the 2 genera *Papio* (baboons) and *Lasiopyga* (grivets). Barbary apes and the various species of macaque, on the other hand, gave unsatisfactory results. Stewart in his experiments has adopted the wise precaution of examining the conjunctivae of each animal for evidence of spontaneous folliculosis before inoculation. He also advocates that suitable animals should first be tested by injection with nontrachomatous material prior to experimental inoculation. According to Stewart, the signs of disease seen in a susceptible animal are as follows:

> "Between 20 to 30 days after inoculation, the conjunctiva of the fornix is found covered with follicles, small, clear, blister-like projections about 1 mm. in diameter. They extend down to the upper edge of the tarsus but not further. When the animal is at rest they are clear, white or colourless and resemble drops of dew, if the animal struggles, however, the small vessels in their core fill with blood and they then look like drops of red jelly. The surface of the conjunctiva may be congested or not. There is no discharge and the animal gives no signs of feeling the condition. In a few cases early symptoms occur such as congestion, oedema, or false membrane formation either on the second day or after an interval, about the 10th to the 18th day."

Stewart regards the result of an experimental inoculation as positive only when follicles appear about the 20th to 30th day and cover the whole conjunctiva of the upper fornix. Control inoculations with certain nontrachomatous materials such as bacteria, and irritants like kaolin, failed to produce follicles on the healthy conjunctiva.

Bland (1944 *a*) reported that the grivet was susceptible, but that inclusions could not be demonstrated, they could be found, however, in men inoculated with grivet material. Bland (1944 *a, b*) stressed that baboons, grivets, and vervets are all prone to develop spontaneous folliculosis, identical in appearance with the changes induced by trachoma.

Cervicitis has been produced in baboons (Braley, 1939).

The susceptibility of other animals.

Rabbits. According to Julianelle and Harrison (1937), rabbits can be infected with the virus when inoculated intratesticularly. These workers obtained scrapings of conjunctival tissue from human cases, emulsified them in veal infusion broth, and then injected 0.5 c.c. of the fluid into each testicle of 2 rabbits. The first rabbit was killed after 1 week, the testicles were removed, emulsified in saline, and the material inoculated into the conjunctival surface of a rhesus monkey. A week later the animal developed follicles on its eyelid which persisted for 1 to 2 months. The testicle of the second rabbit was excised 2 weeks after inoculation, and the material was likewise introduced into another monkey. The result in the second animal suggested that the virulence of the virus had increased, since the monkey showed trachomatous lesions after a shorter incubation period of 5 to 7 days, and these did not heal for several months. By intratesticular passage in rabbits, Julianelle and Harrison (1937) thus claim to have isolated the virus of trachoma by freeing it from extraneous bacteria. No inclusion bodies were, however, found in the conjunctivae of infected monkeys (see also Julianelle, Harrison, and Morris, 1937).

Positive results have also been reported from Brazil by Busacca (1937 *a*) who inoculated material into the vitreous of rabbits and produced lymphocytic deposits in various organs. Micro-organisms resembling *Rickettsiae* were also found in the tunica vaginalis of the rabbit, in preparations stained with Victoria blue.

Efforts to adapt the virus to rabbits by repeated intracerebral inoculation have

been negative, according to Weiss (1933 *c*). Krümmel (1937) claims to have transmitted the infection from the human being to the conjunctiva of the rabbit.

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trachoma, only 50 per cent of them gave a positive reaction, while among 111 non-trachomatous control subjects, 43 per cent. gave a positive result.

The results of the Tricoire test, therefore, appear to be disappointing.

CHEMOTHERAPY IN TRACHOMA

A number of workers has reported favorably on the use of sulfonamides, given orally, parenterally, or locally (Sie Boen Lian, 1938, Burnet, Cuénod, and Nataf, 1939; MacCallan, 1939, *Rev. internat du Trachome*, 1939, *16*, 137, Giller, 1940; Lee and Rottenstein, 1940, Miterstein and Stern, 1945; Sorsby, 1945; Thygeson. 1945). Some claims have also been made for penicillin (Darius, 1945, Thygeson, 1945).

In vitro, sulfonamide has no appreciable effect on the infectivity of virus (Julianelle and Smith, 1942). It is difficult to say whether the effect of chemotherapeutic agents is chiefly on the virus or on secondary invaders.

RELATIONSHIP OF TRACHOMA TO OTHER VIRUSES

It appears that the viruses of trachoma and inclusion conjunctivitis are closely related, and this subject is discussed on p 720.

The studies of Rake, Shaffer, and Thygeson (1942) suggest that antigenic components are shared between the virus of trachoma and the members of the LGI-psittacosis-pneumonitis group (see p. 720).

RELATIONSHIP OF TRACHOMA VIRUS TO *RICKETTSIAE*

There is no doubt that some of the morphological forms of trachoma virus present a superficial resemblance to *Rickettsiae*, and Busacca (1935) has, in fact, proposed the term *Rickettsia trachome*. Certain workers have advanced the view that trachoma virus is really rickettsial in nature (Busacca, 1937 *b*, Cuénod and Nataf, 1937 *c*, Poleff, 1937, 1938, Foley and Parrot, 1938). Venable and Pollock (1943) have published a general review of the question of the relationship of trachoma to *Rickettsiae*.

A number of other workers, with whom we agree, do not accept that the trachoma inclusions are strictly identical with *Rickettsiae* (Thygeson, 1938 *b*, Baltazard, personal communication, Gonnert and Wohlrab, 1942)

As further support for the relationship between trachoma virus and *Rickettsiae*, Poleff (1936 *a, b*) claimed to have obtained an abundant growth of rickettsia-like organisms in tissue culture (see also 1939)

Then, positive Weil-Felix reactions have been demonstrated in the sera of trachoma cases (Poleff and Nain, 1938, Postić, 1939, Gomes and Jordão, 1940) Poleff and Nain have also demonstrated skin reactions to heated *Proteus* OX19 antigen It seems to be generally agreed, however, that the results of Weil-Felix tests are so variable and inconclusive as to be of no value from the diagnostic point of view, there is no evidence from this test that the causal agent of trachoma is rickettsial in origin (Kirk, McKelvie, and Drysdale, 1939, Manditch, 1939, Reis, 1939, Goreczky and von Grósz, 1941).

Additional evidence concerns the alleged transmission of trachoma by lice Cuénod and Nataf (1936, 1937 *a*) claimed that the virus multiplied rapidly in the louse without losing its pathogenicity for man or ape They have suggested (1937 *d*) that the disease is spread by rubbing the eyelids with fingers contaminated with crushed lice (see also Foley and Parrot, 1937 *b*) However, Trapesontzewa (1939) and Weigl (1939) failed to demonstrate *Rickettsiae* in lice.

It does not appear, therefore, that there is much justification for regarding the trachoma virus as a *Rickettsia*

BIOLOGICAL THERAPY IN TRACHOMA

Autohemotherapy has been tried by Fehmi (1931) and found to give but little relief, but Lerner (1937) practiced the same method and after injecting 10 c c. of

blood every other day until 15 doses were administered, reported improvement in early cases

A substance known as "trachocid," devised by Brecher (1935), consisting of bee venom and snake venom which were rendered atoxic and nonirritant by a chemical process, has been recommended by Lobel (1936) Attempts to immunize patients against the disease have been made by Derkač (1937), who excised fragments of tissue from the conjunctivae of children and then transplanted the material into the subcutaneous surface of the forearm Derkač states that this procedure modified the course of the disease which subsided in a few months, and, furthermore, that individuals so treated acquired immunity to reinfection.

Esteban (1933) reports good results with local immunotherapy, and has used a vaccine prepared after the method originally employed by Pietro Vancea, consisting of a saline emulsion of trachomatous granulation tissue which had been heated to 60° C. for 30 minutes Galeazzi (1934) and Ruata (1934) have used a bacterial vaccine consisting of organisms isolated from the conjunctival sac, Krylov and Rostovtsev *(1936) reported poor results when using brilliant green,* which was not as effective as copper sulfate.

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A number of other workers, with whom we agree, do not accept that the trachoma inclusions are strictly identical with *Rickettsiae* (Thygeson, 1938 *b*, Baltazard, personal communication, Gonnert and Wohlrab, 1942).

As further support for the relationship between trachoma virus and *Rickettsiae*, Poleff (1936 *a*, *b*) claimed to have obtained an abundant growth of rickettsia-like organisms in tissue culture (see also 1939).

Then, positive Weil-Felix reactions have been demonstrated in the sera of trachoma cases (Poleff and Nain, 1938, Postić, 1939, Gomes and Jordão, 1940) Poleff and Nain have also demonstrated skin reactions to heated *Proteus* OX19 antigen It seems to be generally agreed, however, that the results of Weil-Felix tests are so variable and inconclusive as to be of no value from the diagnostic point of view, there is no evidence from this test that the causal agent of trachoma is rickettsial in origin (Kirk, McKelvie, and Drysdale, 1939, Manditch, 1939, Reis, 1939, Goreczky and von Grósz, 1941)

Additional evidence concerns the alleged transmission of trachoma by lice Cuénod and Nataf (1936, 1937 *a*) claimed that the virus multiplied rapidly in the louse without losing its pathogenicity for man or ape They have suggested (1937 *d*) that the disease is spread by rubbing the eyelids with fingers contaminated with crushed lice (see also Foley and Parrot, 1937 *b*) However, Trapesontzewa (1939) and Weigl (1939) failed to demonstrate *Rickettsiae* in lice.

It does not appear, therefore, that there is much justification for regarding the trachoma virus as a *Rickettsia*

BIOLOGICAL THERAPY IN TRACHOMA

Autohemotherapy has been tried by Fehmi (1931) and found to give but little relief, but Lerner (1937) practiced the same method and after injecting 10 c c of

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Inclusion Urethritis of the Male

Inclusion bodies and elementary bodies resembling those found in inclusion blennorrhea have also been detected in the male urethra Lindner (1909) reported their presence in cases of urethritis studied by him, in which gonococci were absent (see also Fritsch, Hofstaetter, and Lindner, 1910, and Heymann, 1913). Thygeson and Mengert (1936) also found inclusion bodies in a man who developed subacute urethritis 7 days after sexual intercourse, which later subsided and gave rise to a chronic intermittent discharge which lasted 7 months before it healed completely. In this case inclusions were found in urethral smears made 3 months after the onset of symptoms

Harrison and Worms (1939) discuss the rôle of the virus in the causation of cases of "nonspecific urethritis" (see also Meenan, 1947).

INFECTION OF THE RESPIRATORY TRACT

Infections of the upper respiratory passages due to this virus have been reported by Morax (1933), Aust (1933), and Poelchau (1937). The accurate diagnosis of such cases is, however, a difficult problem, as inclusion bodies cannot easily be found (Thygeson and Mengert, 1936) Moreover, if they are found a certain amount of care is necessary in the interpretation of the result, owing to the fact that Broadhurst *et al* (1936) have demonstrated inclusion bodies in films of saliva obtained from the mouth, throat, and other parts of the respiratory tract of normal persons

ACCIDENTAL INFECTIONS

Infection has occurred in doctors and nurses accidentally infected with secretions or blood during gynecological operations or eye examinations (Thygeson and Mengert, 1936, Julianelle, 1937).

MORBID HISTOLOGY

The histopathological lesions of blennorrhea neonatorum have been studied by Wolfrum (1910), who came to the conclusion that the absence of follicles in chil-

trachomatous follicles by their small size and the absence of a circumscribed band of fibrous tissue surrounding them Healed lesions of inclusion conjunctivitis, moreover, show no cicatricial tissue formation, whereas this invariably occurs as the end result of trachoma Thygeson and Mengert (1936) studied biopsy tissue excised from the lower fornix in a case of inclusion conjunctivitis, 6 weeks after its onset. In sections they found that the epithelium was of normal thickness, but was heavily infiltrated with polymorphonuclear leukocytes, together with diffuse subepithelial infiltration with plasma cells and numerous lymphocytes. There were also present numerous characteristic follicles with avascular centers, composed of large mononuclear cells surrounded by plasma cells and lymphocytes

EPIDEMIOLOGY

The infection, in its various clinical forms, has a wide distribution in Germany, Austria, France, various other parts of Europe, Russia, Japan, Australasia, and N. America (Heymann, 1909, Lindner, 1909, 1910, 1911, Noguchi and Cohen, 1911, Huntemueller and Paderstein, 1913, Axenfeld, 1914, Brown, 1914, Williams, 1914;

CHAPTER LXVI

INFECTION WITH THE VIRUS OF INCLUSION CONJUNCTIVITIS

This virus most commonly causes infection of the conjunctiva in newborn babies (virus ophthalmia neonatorum), it also causes conjunctivitis in older persons, and this is frequently contracted in swimming pools It is also responsible for a mild form of cervicitis and urethritis. The distinguishing feature of the infection is the presence of basophilic intracytoplasmic inclusions in epithelial cells, identical with those described in trachoma (Halberstaedter and Prowazek, 1909, 1910, Heymann, *1909, Stargardt, 1909; Herzog, 1910).*

CLINICAL FEATURES OF CONJUNCTIVITIS

The incubation period in the newborn infant is 5–9 days, in the adult the incubation is usually about 7 days (Thygeson, 1934).

The clinical features, which are not particularly distinctive, are as follows (see, e g, Noguchi and Cohen, 1911; *Gradle, 1916;* Lumbroso, 1933; McKee, 1935, *Thygeson and Mengert, 1936, Julianelle, 1937).*

There is edema of the conjunctivae, with mucopurulent exudate. The conjunctivae become granular, due to the formation of translucent follicles. The preauricular glands may be swollen and tender. The illness usually lasts for 3–6 months Cicatrization is most unusual, thus distinguishing the condition from trachoma, which is otherwise closely similar.

INVOLVEMENT OF THE GENITO-URINARY TRACT

Inclusion Cervicitis of the Female

The evidence shows that in the case of newborn children who develop the disease, the infection is acquired at birth from the infected genital passages of the mother.

Thus, Halberstaedter and Prowazek (1909, 1910) and Heymann (*1910*) found inclusions in epithelial cells derived from the genital tract of mothers whose children *had developed the disease shortly after birth.*

Thygeson and Mengert (1936) likewise obtained similar results and, in a series of excellent microscopic preparations, they clearly demonstrated the presence of numerous elementary bodies in scrapings obtained from the cervix uteri of infected mothers Vaginal smears, on the other hand, yielded less satisfactory results, for although the virus could be found in the material examined, the detection of cell inclusions proved to be an exceedingly difficult task, owing to the density of the cellular and bacterial content of the vaginal exudate These workers, therefore, concluded from their studies that the cells with inclusions found in vaginal secretion were merely desquamated cells that had been shed from the epithelial covering of the cervix uteri which was the active focus of infection Hamburger (*1934*), on the contrary, concluded that the virus multiplied in the vagina and not in the cervix. He also expressed the opinion that inclusion vulvitis should be recognized as a clinical entity.

The epithelial cells involved are the transitional cells just within the external os (Thygeson and Stone, *1942 a)*

It should be realized that inclusions may be only scanty in the cervical scrapings of mothers of babies with virus ophthalmia, and may not be found in more than a small percentage of cases where infection is present (Sorsby, Hoffa, and Young. 1944)

by Thygeson and Mengert (1936), neither have they been able to detect the existence of a stainable matrix. Thygeson and Mengert observe, however, that the inclusion cavity "is probably filled with a fluid which has slight agglutinating properties," so that inclusion bodies which have been extruded from cells consist of clumps of elementary bodies, which remain as compact masses. It was also noted by the same 2 observers that the virus readily entered the epithelial cells of the conjunctiva, cervix, or urethra, but never attacked the keratinized transitional epithelial cells of the adult vagina or that found at the margin of the eyelids

Life Cycle of the Virus

This has been carefully studied by Thygeson (1934), who did so by artificially transferring infection from the affected eye to the normal eye of a patient, after which he obtained scrapings from the lower tarsal conjunctiva at regular intervals, and in this manner was able to study the developmental phases of the virus

Stained films made from scrapings obtained before inoculation showed no inclusion bodies, while after infection he observed the following changes

"30 hours Conjunctiva normal Scrapings revealed numerous inclusions, 99 per cent. being of the elementary-body type, the remaining 1 per cent being of mixed type There were no leucocytes

"51 hours Conjunctiva normal Numerous inclusions, 99 per cent being of early initial-body type A few free elementary bodies and an occasional polymorphonuclear neutrophile

"75 hours Conjunctiva normal. Numerous inclusions, mostly of mixed type, with an occasional elementary-body type

"100 hours Conjunctiva normal Numerous inclusions, mainly of elementary-body type. A few very early initial-body inclusions Increased leucocytic reaction

"122 hours Inflammation and secretion were beginning Inclusions of initial to mixed type predominated Many polymorphonuclear leucocytes

"144 hours Inflammation and secretion marked Mixed inclusions predominated A few elementary-body inclusions The cycle had now become somewhat indistinct.

"165 hours Majority of inclusions of elementary-body type with a few of the initial-body type."

From the above results it was concluded that the complete cycle of development from the elementary body stage back to the same stage was observed during the first 6 days of incubation It thus appears that the life cycle of the inclusion blennorrhea virus is approximately 48 hours, which is of particular interest since it coincides with the duration of the life cycle of psittacosis virus (Bedson, 1933).

Attempts to Cultivate the Virus

Noguchi and Cohen (1913, 1914) claimed to have been able to cultivate the inclusion bodies found in this disease, but the work has lacked confirmation. Efforts to grow the virus on the chorio-allantoic membrane of the developing chicken embryo have been unsuccessful (Thygeson and Mengert, 1936).

Transmission to Man

Man can be infected by inoculating the conjunctival sac (Thygeson, 1934; Allen, 1944).

Inoculation of Animals

Lindner (1909) injected secretion from a human case of inclusion conjunctivitis into the eyelid of a *Macacus rhesus* monkey, and reproduced a granulomatous condition

Later, Fritsch, Hofstaetter, and Lindner (1910) similarly infected monkeys with conjunctival secretion obtained from infected infants, as well as the vaginal secretion derived from the mothers of these children. They also obtained positive re-

Comberg, 1920; Chaillous and Nida, 1922, Kalt, 1922; Morax, 1922, 1933; Engelking, 1925, Paderstein, 1925; Rohrschneider, 1926; Sandmann, 1926; Bahn, 1927; Fodor, 1929, Rötth, 1932, Thygeson, 1934; Kankrov, 1935; Rameer, 1935; Rutherford, 1935; Fujita and Nagashige, 1936, Thygeson and Mengert, 1936; Howard, 1938, Julianelle, Harrison, and Lange, 1938; Thygeson and Stone, 1942 a, Derrick, 1943; Williams, 1946).

In Britain, cases of ophthalmia neonatorum have been described (Sorsby, Hoffa, and Young, 1944), but investigations that we made in London did not suggest that inclusion cervicitis or urethritis was anything but exceedingly rare in patients attending V. D. clinics.

A reservoir of infection is afforded by mild cases of inclusion cervicitis and urethritis, these infections being transmitted venereally between adults. Babies born to mothers harboring the virus in the cervix may develop virus ophthalmia. Swimming-pool conjunctivitis is contracted by fouling of the water with urine. Infection may also be transmitted mechanically, as by infected eye instruments, or accidentally, as in doctors or nurses.

PROPERTIES OF THE VIRUS

Filtrability and Size of the Virus

Botteri (1912) and Gebb (1914) proved that the infective agent of the disease was capable of traversing the pores of earthenware filters. The virus is filtrable through Elford's collodion filters of APD 0.65 μ (Thygeson, 1934; Julianelle, Harrison, and Lange, 1938, Julianelle, 1940). Stained EB's measure 0.25 μ, and initial bodies 0.3–0.8 μ (Thygeson, 1934; Tilden and Gifford, 1936).

Morphology and Staining Reactions

Films prepared from conjunctival secretion or scrapings should be made on clean glass slides and stained with dilute Giemsa's stain, according to any one of the methods described in Ch. III. Rivers's modification of Castaneda's method has been recommended by Thygeson and Mengert (1936). The elementary bodies of inclusion conjunctivitis virus stain poorly with aniline dyes and are gram negative in reaction [illegible]
(Fodor, 1935 [illegible]
no different [illegible]
[For a good illustration of these bodies see Heymann (1913).]

The inclusion bodies of inclusion conjunctivitis are now regarded by Thygeson and other workers as intracellular colonies of the virus.

Appearance of the Virus when Seen by Darkfield Illumination

The elementary and initial bodies appear as highly refractile dots of light when viewed by this method. They are comparatively uniform in shape and size and present a characteristic appearance when examined by oblique light. Even though the bodies present a striking picture by this method, we advise that all films should first be stained and examined by direct light, and then, only if elementary bodies are thought to be present, should their existence be confirmed by darkground examination (see van Rooyen, 1937).

Multiplication and Development of the Virus in Tissues

The successive stages exhibited by the inclusion conjunctivitis virus during its growth in conjunctival epithelial cells are similar to those described by Lindner (1910). Thus, the virus colony develops at the expense of the cell cytoplasm, and in so doing forms a vacuole which is first filled by initial bodies and later by elementary bodies, which begin by appearing from the center of the mass.

The bridge or dumb-bell forms described by Lindner have not been observed

by Thygeson and Mengert (1936), neither have they been able to detect the existence of a stainable matrix. Thygeson and Mengert observe, however, that the inclusion cavity "is probably filled with a fluid which has slight agglutinating properties," so that inclusion bodies which have been extruded from cells consist of clumps of elementary bodies, which remain as compact masses It was also noted by the same 2 observers that the virus readily entered the epithelial cells of the conjunctiva, cervix, or urethra, but never attacked the keratinized transitional epithelial cells of the adult vagina or that found at the margin of the eyelids

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... been extruded from cells consist of
... as compact masses It was also noted
by the same 2 observers that the virus readily entered the epithelial cells of the conjunctiva, cervix, or urethra, but never attacked the keratinized transitional epithelial cells of the adult vagina or that found at the margin of the eyelids

Life Cycle of the Virus

This has been carefully studied by Thygeson (1934), who did so by artificially transferring infection from the affected eye to the normal eye of a patient, after which he obtained scrapings from the lower tarsal conjunctiva at regular intervals, and in this manner was able to study the developmental phases of the virus

Stained films made from scrapings obtained before inoculation showed no inclusion bodies, while after infection he observed the following changes

"30 hours Conjunctiva normal Scrapings revealed numerous inclusions, 99 per cent being of the elementary-body type, the remaining 1 per cent being of mixed type. There were no leucocytes

"51 hours Conjunctiva normal Numerous inclusions, 99 per cent being of early initial-body type A few free elementary bodies and an occasional polymorphonuclear neutrophile

"75 hours *Conjunctiva normal* Numerous *inclusions, mostly of mixed type, with an* occasional elementary-body type.

"100 hours Conjunctiva normal Numerous inclusions, mainly of elementary-body type. A few very early initial-body inclusions Increased leucocytic reaction

"122 hours Inflammation and secretion were beginning Inclusions of initial to mixed

...

body type"

From the above results it was concluded that the complete cycle of development from the elementary body stage back to the same stage was observed during the ... life cycle of the inclusion blen- ... *of particular interest since it co-* ... acosis virus (Bedson, 1933).

Attempts to Cultivate the Virus

Noguchi and Cohen (1913, 1914) claimed to have been able to cultivate the inclusion bodies found in this disease, but the work has lacked confirmation Efforts to grow the virus on the chorio-allantoic membrane of the developing chicken embryo have been unsuccessful (Thygeson and Mengert, 1936)

Transmission to Man

Man can be infected by inoculating the conjunctival sac (Thygeson, 1934, Allen, 1944)

Inoculation of Animals

Lindner (1909) injected secretion from a human case of inclusion conjunctivitis into the eyelid of a *Macacus rhesus* monkey, and reproduced a granulomatous condition

Later, Fritsch, Hofstaetter, and Lindner (1910) similarly infected monkeys with conjunctival secretion obtained from infected infants, as well as the vaginal secretion derived from the mothers of these children *They also obtained* positive re-

Comberg, 1920, Chaillous and Nida, 1922; Kalt, 1922, Morax, 1922, 1933, Engelking, 1925, Paderstein, 1925; Rohrschneider, 1926; Sandmann, 1926, Bahn, 1927, Fodor, 1929, Rotth, 1932; Thygeson, 1934; Kankrov, 1935, Rameer, 1935, Rutherford, 1935; Fujita and Nagashige, 1936; Thygeson and Mengert, 1936; Howard, 1938, Julianelle, Harrison, and Lange, 1938; Thygeson and Stone, 1942 *a*, Derrick, 1943, Williams, 1946).

In Britain, cases of ophthalmia neonatorum have been described (Sorsby, Hoffa, and Young, 1944), but investigations that we made in London did not suggest that inclusion cervicitis or urethritis was anything but exceedingly rare in patients attending V. D. clinics

A reservoir of infection is afforded by mild cases of inclusion cervicitis and urethritis, these infections being transmitted venereally between adults. Babies born to mothers harboring the virus in the cervix may develop virus ophthalmia. Swimming-pool conjunctivitis is contracted by fouling of the water with urine. Infection may also be transmitted mechanically, as by infected eye instruments, or accidentally, as in doctors or nurses.

PROPERTIES OF THE VIRUS

Filtrability and Size of the Virus

Botteri (1912) and Gebb (1914) proved that the infective agent of the disease was capable of traversing the pores of earthenware filters. The virus is filtrable through Elford's collodion filters of APD 0 65 μ (Thygeson, 1934, Julianelle, Harrison, and Lange, 1938; Julianelle, 1940). Stained EB's measure 0.25 μ, and initial bodies 0 3–0.8 μ (Thygeson, 1934; Tilden and Gifford, 1936).

Morphology and Staining Reactions

Films prepared from conjunctival secretion or scrapings should be made on clean glass slides and stained with dilute Giemsa's stain, according to any one of the methods described in Ch. III. Rivers's modification of Castaneda's method has been recommended by Thygeson and Mengert (1936). The elementary bodies of inclusion conjunctivitis virus stain poorly with aniline dyes and are gram negative in reaction The inclusions can also be demonstrated by supravital staining methods (Fodor, 1935) Morphologically, the inclusion bodies present in this condition are no different from the Halberstaedter-Prowazek inclusion bodies found in trachoma. [For a good illustration of these bodies see Heymann (1913).]

The inclusion bodies of inclusion conjunctivitis are now regarded by Thygeson and other workers as intracellular colonies of the virus.

Appearance of the Virus when Seen by Darkfield Illumination

The elementary and initial bodies appear as highly refractile dots of light when viewed by this method. They are comparatively uniform in shape and size and present a characteristic appearance when examined by oblique light Even though the bodies present a striking picture by this method, we advise that all films should first be stained and examined by direct light, and then, only if elementary bodies are thought to be present, should their existence be confirmed by darkground examination (see van Rooyen, 1937)

Multiplication and Development of the Virus in Tissues

The successive stages exhibited by the inclusion conjunctivitis virus during its growth in conjunctival epithelial cells are similar to those described by Lindner (1910). Thus, the virus colony develops at the expense of the cell cytoplasm, and in so doing forms a vacuole which is first filled by initial bodies and later by elementary bodies, which begin by appearing from the center of the mass

The bridge or dumb-bell forms described by Lindner have not been observed

and animal pathogenicity It would appear, nevertheless that some antigenic components are shared by the two groups, as convalescent sera may fix complement with LGI-psittacosis antigens (Rake, Shaffer, and Thygeson, 1942).

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sults with urethral secretion derived from male cases of inclusion urethritis, in which the presence of gonococci had been excluded by careful bacteriological examination These workers succeeded in transmitting the infection from one monkey to another, and demonstrated the presence of inclusion bodies and free elementary bodies in conjunctival scrapings prepared from the infected animals. Baboons were also found to be susceptible to infection.

Heymann (1910, 1913) reproduced conjunctivitis in baboons and cercopithecus monkeys, using conjunctival secretion obtained from infected children and also material from the genitalia of their parents Thygeson (1934) and Thygeson and Mengert (1936) likewise reproduced inclusion conjunctivitis in monkeys, and found that the sphinx baboon was more susceptible to infection than the *Macacus rhesus* monkey. They were also able to produce cervicitis in female baboons, 8 to 12 days after the intrauterine inoculation of infected vaginal secretion from human cases Julianelle (1940), and Julianelle, Harrison, and Lange (1938), confirm the susceptibility of rhesus monkeys to conjunctival inoculation.

The rabbit, guinea-pig, white rat, pig, and *dog* are resistant to experimental infection (Thygeson, 1934).

IMMUNITY

There appears to be no general immunity following infection with inclusion conjunctivitis virus Noguchi and Cohen (1911) have recorded the occurrence of relapses or reinfections, and a number of workers have drawn attention to the fact that mothers who carry the virus are liable to infect their own eyes with the organism (James, 1930; Aust, 1933, and Thygeson, 1934). There would appear to be no neutralizing antibodies in the sera of individuals who have recovered from one attack of inclusion conjunctivitis. Thygeson and Mengert (1936) clearly demonstrated that convalescent patients' sera failed to neutralize the virus *in vitro,* and that baboons inoculated with a mixture of the two developed the disease in due course Efforts to agglutinate the virus also failed, and these workers suggest that the localization of the virus in epithelial tissues may conceivably have been responsible for the absence of agglutinins in the patients' sera Thygeson's view is of interest to ourselves, since during the course of our own studies on the elementary bodies of molluscum contagiosum (van Rooyen, 1938) we too failed to detect the presence of agglutinins in the sera of patients, and attributed their absence to a similar cause

SPECIFIC TREATMENT

Oral or local sulfonamides are said to be effective in man or monkey, inclusions disappearing rapidly (Giddens and Howard, 1940, Thygeson, 1941, Thygeson and Stone, 1942 *b*) Local penicillin is also of value (Sorsby, 1945)

RELATIONSHIP TO TRACHOMA

Clinically, inclusion conjunctivitis is mild and ends in complete recovery, whereas trachoma leads to permanent cicatrization In the early stages the two conditions are, however, closely similar

The two agents are microscopically indistinguishable, and Lindner (1925, 1935 *a, b, c*) has regarded the viruses of trachoma and "paratrachoma" (inclusion conjunctivitis) as identical The viruses are evidently closely related, yet in infected monkeys no cross protection can be demonstrated, indicating differing antigenic structures (Julianelle, 1940).

RELATIONSHIP TO LGI-PSITTACOSIS

Morphologically the trachoma and inclusion conjunctivitis viruses resemble fairly closely members of the LGI-psittacosis group There are, however, many points of biological difference between the two, particularly as regards cultivation

CHAPTER LXVII

INTRODUCTION: THE PNEUMONITIS-PSITTACOSIS-LGI GROUP[1]

THE MEMBERS of this group have common biological properties that separate them somewhat from other viruses. In certain respects the members of this group may be considered as intermediate between *Rickettsiae* and viruses, although classifiable as viruses. Thus they are all relatively large, and are readily stained with simple basophilic dyes (so-called Castañeda positive). They form large basophilic inclusions in endothelial and epithelial cells, and grow well in the yolk sac. They infect a wide range of experimental animals, and some cause infection of birds. Certain viruses produce what appears to be an endotoxin, and some are sensitive to sulfonamides and antibiotics.

The best known agents in the group are. pneumonitis viruses, such as meningo-pneumonitis (MP), Baker's feline pneumonitis, pneumonia virus of mice (PVM), Nigg's mouse pneumonitis virus, SF, and Louisiana viruses (Ch LXVIII), psittacosis and ornithosis (Ch LXX and LXXI), lymphogranuloma inguinale (Ch LXXII). It is convenient also to discuss in this section the question of atypical pneumonia, the etiology of which is not yet clarified (Ch LXIX)

BIOLOGICAL PROPERTIES

In general, the effects produced in experimental animals and eggs are similar. However, there are definite differences between the Castañeda positive viruses,

TABLE 31

SOME DIFFERENTIAL CHARACTERISTICS OF THE PNEUMONITIS-PSITTACOSIS-LGI GROUP
(See Beck, Eaton, and O'Donnell, 1944; Hamre and Rake, 1944)

	Mice				*Cotton Rats*	*Hamsters*		*Guinea-pigs*		*Susceptibility to Sulfonamides*
	Nasal	*Venous*	*Peritoneal*	*Cerebral*	*Nasal*	*Nasal*	*Cerebral*	*Cerebral*	*Peritoneal*	
LGI	+	o	o, nc	+c	+	±	±		o	sensitive
Mouse pneumonitis	+	+	o, nc	o	+	+			o	sensitive
Meningopneumonitis	+	+	±c	+	+	+	+	±	±	o
Feline pneumonitis	+	+	±	±						o
Psittacosis	+		+c	+	+	+	±	±	o	o
Ornithosis	+		±c	+	+	+				
SF	+		o, nc	+c	+	+	+	±	±	

+ = generally fatal
o = no effect
± = occasionally fatal
c = carrier stage
nc = no carrier stage

[1] References are appended at the conclusion of Ch LXIX, p 733 et seq

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using sera prepared in roosters (St. John and Gordon, 1947). In this work, in addition to the better known viruses, study was made of Ann Arbor virus and Illinois virus Cross neutralization tests were performed with rooster antisera and virus suspensions, the test mixtures being inoculated in mice nasally or cerebrally according to the virus. The results, including those of Hilleman (1945) may be tabulated as follows:

TABLE 32

ANTIGENIC RELATIONSHIPS IN THE PNEUMONITIS PSITTACOSIS GROUP AS SHOWN BY VIRUS NEUTRALIZATION TESTS

Viruses	Antisera					
	Feline Pneumonitis	*Ann Arbor (Kempf)*	*Ann Arbor (St John)*	*Mouse Pneumonitis*	*LGI*	*MP*
Feline pneumonitis	+	o		o	o	o
Ann Arbor .	o	+	+	+	o	o
Mouse pneumonitis (Chicago)	o	±	±	+	o	o
Mouse pneumonitis (Atherton)				+		o
LGI	o		o	o	+	o
MP	o	+	o	o	o	+
Ornithosis .				o		+
Psittacosis . .	o			o	o	o
SF . .	o			o	o	o
Illinois .	o			o	o	o

+ = neutralization ± = partial o = no neutralization

These tests show that each virus studied, except Ann Arbor, possesses a distinctive antigen Ann Arbor seems to share a common antigen with mouse pneumonitis Meningopneumonitis and ornithosis antigens appear to be identical

CONCLUSIONS

We may conclude that the viruses named above are antigenically related, yet are mostly distinct entities Probably further work will show antigenic relationships between these pneumonitis.
It is eviden and antigenic relationships or es as follows (1) lymphogranuloma inguinale, (2) psittacosis, ornithosis, and meningopneumonitis; (3) SF, Louisiana, Baker's virus, Ann Arbor, and mouse pneumonitis

and by the choice of suitable animals and routes of inoculation it is often possible to be reasonably certain of the correct classification of an unknown basophilic virus Some of the differential tests that may be used are indicated in Table 31. In addition, use may be made of pigeons and other birds, complement fixation, cross immunity and cross neutralization tests, especially in mice (see below). Study of any toxin produced may also be of value.

ANTIGENIC RELATIONSHIPS

The question of the antigenic relationship between the members of the group has been investigated by various means, particularly by complement fixation, virus neutralization, and active immunity tests. Cross reactions are more commonly observed by the complement fixation reaction, and are less commonly demonstrated by virus neutralization or cross resistance tests.

Complement fixation tests with virus antigens and human or animal sera suggest that the following v

components psittacosis,

ana virus, SF, and mouse

Corey, 1942, Eaton, Martin, and Beck, 1942; Smadel, Wertman, and Reagan, 1943, Beck, Eaton, and O'Donnell, 1944; Florman, 1945, Larson and Olson, 1946).

The relation between certain of these viruses is closer than between others, and various observations may be quoted.

1. Psittacosis antisera will fix complement with ornithosis and LGI antigens (Levine, Holder, and Bullowa, 1943; Smadel, Wall, and Gregg, 1943; Larson and Olson, 1946) Psittacosis and ornithosis are closely related, but can be differentiated by active immunity tests in mice (Beck, Eaton, and O'Donnell, 1944).

2 The sera of LGI cases will fix complement with antigens prepared from the following viruses

Baker's virus . ..	... Hamre and Rake, 1944; Vilches, Parodi, and Chialvo, 1946
Ornithosis ..	. Smadel, Wertman, and Reagan, 1943, Bowser and Nigg, 1946
Psittacosis	Smadel, Wertman, and Reagan, 1943; Levine, Holder, and Bullowa, 1943, De Burgh, Jackson, and Williams, 1945, Bowser and Nigg, 1946
Louisiana . .	Larson and Olson, 1946
Meningopneumonitis ..	Eaton, Martin, and Beck, 1942

3 A rise in titer to both LGI and meningopneumonitis viruses has been recorded in the sera of cases of pneumonia due to SF and ornithosis viruses (Eaton, 1945). The sera of pigeons infected with ornithosis proved rather more specific, and fixed complement with meningopneumonitis, but not with LGI (Eddie and Francis, 1942) The close relationship between ornithosis and meningopneumonitis has been confirmed by CF tests by Larson and Olson (1946), and in virus neutralization tests in mice using fowl sera (Hilleman and Gordon, 1944, Hilleman, 1945, St. John and Gordon, 1947)

4. The SF virus is related to, but can be separated from, ornithosis, it is distinct from meningopneumonitis, psittacosis, and lymphogranuloma (Beck and Eaton, 1942, Beck, Eaton, and O'Donnell, 1944, Meiklejohn, Beck, and Eaton, 1944).

5. Sera to Louisiana virus were found to fix complement with psittacosis, ornithosis, and LGI viruses, but mice and guinea-pigs immunized by killed meningopneumonitis and psittacosis viruses were not resistant to Louisiana virus (Larson and Olson, 1946).

6 Baker's virus is related antigenically to psittacosis and mouse pneumonitis (Thomas and Kolb, 1943), but in virus neutralization tests with fowl sera is seen to occupy a distinctive position without showing cross reactions (Hilleman and Gordon, 1944, Hilleman, 1945)

7 More specific differentiation has been achieved by virus neutralization tests,

gray-lung rodent virus of Andrewes and Glover (1945), and an agent causing lesions in guinea-pigs (Lépine, Sautter, and Lamy, 1943; Lépine and Sautter, 1945).

PNEUMONITIS VIRUSES PROBABLY INFECTING MAN

Meningopneumonitis (MP) Virus

This virus was isolated from ferrets inoculated with throat washings from patients suffering from an influenza-like disease (Francis and Magill, 1938). It has been isolated from a fatal case of AP by Meiklejohn, Beck, and Eaton (1944).

1. The EBs are basophilic and measure 354 ± 41 mμ in electron micrographs (Kurotchkin *et al*, 1947).

2. It infects mice and ferrets nasally, and monkeys and mice cerebrally Rabbits can be infected tracheally (Morgan, 1946), cotton rats, hamsters, and guinea-pigs are also susceptible (see Table 31).

3. The virus grows in the yolk sac, and produces a toxin killing mice rapidly on intravenous injection. Antitoxic sera can be prepared, and are highly specific (Rake and Jones, 1944). It can also grow in the allantoic sac (Francis and Gordon, 1945).

4. The virus is sensitive *in vivo* to penicillin (Parker and Diefendorf, 1944)

5 As regards sulfonamides, Rake, Jones, and Nigg (1942) did not find any evidence of sensitivity of the virus in mice Rake and Hamre (1944) found that sulfamerazine had no effect on the endotoxin, or on the early deaths produced thereby in mice, however, it prevented later deaths, which are probably due to infection rather than toxemia.

6. Aerosolized virus is sensitive to triethylene glycol (Rosebury *et al.*, 1947).

7. The virus is related to psittacosis, ornithosis, and LGI viruses (see above).

Pneumonia Virus of Mice (PVM)

This virus was isolated by Horsfall and Hahn (1940) from the lungs of apparently healthy mice by passage Horsfall *et al* (1943) recovered 12 strains from patients with AP, all strains were antigenically related to PVM.

1 The virus forms basophilic EBs It measures 100–150 mμ by ultrafiltration (Horsfall and Hahn, 1940, but see also below)

2 The lungs of infected mice contain an agent that agglutinates mouse red cells The agglutination is inhibited by antiserum (Mills and Dochez, 1944). Maximum agglutinability develops after heating the lung extract at 75° C for 5 minutes, the agent is absorbed from active extracts by mouse red cells and fresh normal mouse

3 Curnen and Horsfall (1947), Curnen, Pickels, and Horsfall (1947) have shown that PVM can be separated into free and combined virus. Virus in the combined state, as found in lightly ground lung suspensions, is infectious but does not agglutinate red cells Heat treatment of combined virus releases virus which is noninfective but agglutinates red cells. Free virus is liberated from infected lungs by perfusing prior to removal and then mincing finely, such virus is infectious, and causes hemagglutination Heat treatment renders free virus noninfective, but this still causes agglutination Heat-released virus mixed with lightly ground lung suspension does not give agglutination (artificially combined PVM). The principal hydrated virus particles of naturally free or heat-released PVM were found to have

CHAPTER LXVIII

THE PNEUMONITIS GROUP

A considerable number of viruses have been isolated that have the property of causing pneumonia in man and animals, and are characterized by the formation of basophilic elementary and inclusion bodies.

PNEUMONITIS VIRUSES OF ANIMALS

Nigg's Mouse Pneumonitis Virus

This virus was isolated by Nigg (1942)[1] from apparently normal Swiss mice, and has the following properties

1 The virus forms basophilic EB's The EB's measure 497± 77mμ in electron micrographs (Kurotchkin *et al*, 1947).

2. It is sensitive to sulfonamides (Rake, Jones, and Nigg, 1942, Eaton and Hanford, 1945).

3 Antisera can be prepared in chickens, and cause flocculation of EB's (Hilleman and Gordon, 1943).

4 A toxin is produced when growing in the yolk sac, killing mice rapidly on intravenous injection, antitoxic sera are highly specific (Rake and Jones, 1944).

5 The virus is antigenically related to meningopneumonitis, LGI, and Baker's virus (Thomas and Kolb, 1943, Nigg and Eaton, 1944)

6 The virus is pathogenic for mice, cotton rats, and hamsters (see Table 31).

Baker's Feline Pneumonitis Virus

This virus was isolated by Baker (1942) from a respiratory distemper-like disease of cats

1 It forms basophilic EB's and can be transmitted to mice (see Table 31), rabbits, guinea-pigs, hamsters, and eggs (Baker, 1944). The EB's have been found to measure 466± 78mμ in electron micrographs (Rake *et al*, 1946, Kurotchkin *et al*, 1947) In a later study, Hamre, Rake, and Rake (1947), using purified allantoic fluid, found the EB had a *dense central area, and a surrounding thinner substance, like a membrane, groups of EB* appeared enmeshed in a matrix, the mean diameter was 525± 84mμ

2 The virus is not markedly sensitive to sulfonamides (Hamre and Rake, 1944, 1947, Rake and Hamre, 1944, Eaton and Hanford, 1945) Streptomycin and streptothricin had no effect *in vitro* or *in vivo* on the virus (Hamre and Rake, 1947) They found high concentrations of penicillin active *in vitro*, activity appeared to be associated with some impurity When tested in the yolk sac, penicillin delayed death of the embryos

3 The virus produces a specific toxin which is neutralized only by homologous serum *(Hamre and Rake, 1944, Hamre, Rake, and Rake, 1947) The toxin is not sensitive to* sulfonamides (Rake and Hamre, 1944)

4 Hamre and Rake (1944) point out that the virus can be distinguished from other pneumonitis viruses by its ability to infect mice nasally, intravenously, peritoneally and cerebrally, by its insusceptibility to sulfonamides, and by the specificity of its toxin

5 Its antigenic relationships are discussed above

Miscellaneous Pneumonitis Viruses

A number of viruses producing pneumonia in animals have been described, but have not been studied sufficiently for us to classify them with certainty, although many belong to the basophilic pneumonitis group a virus causing pneumonia and hepatitis in mice (Freeman, 1940), other agents producing pneumonia in mice have been described (Gonnert, 1941, Gross, 1943 *a, b, c*, Karr, 1943, Mooser, 1943, Furth and de Gara, 1944, Thomas and Kolb, 1944, Aufdermaur, 1945, deBurgh, Jackson, and Williams, 1945), a virus producing pneumonia in calves (Baker, 1943), viruses producing pneumonia in hamsters and cotton rats (Pearson and Eaton, 1940, Eaton and van Herick, 1944, Eaton *et al*, 1945), the

[1] References are appended at the conclusion of Ch LXIX, p 733 et seq

3. It grows in the yolk sac

4 It resembles most closely MP and psittacosis viruses, but is distinguished from these by its higher pathogenicity for guinea-pigs, and for mice by the subcutaneous and muscular routes

Miscellaneous Viruses of Presumed Human Origin

A number of basophilic viruses have been isolated by inoculation of material from cases of AP in animals, but for various reasons have not been much studied It is not clear whether they derived from the inoculated animal or the human seed, nor is their relationship to the better known members of the pneumonitis group certain (Reimann, 1938, Herzberg and Gross, 1940, Heinzmann, 1941, Rose and Molloy, 1943).

Weir and Horsfall (1940) isolated a virus by inoculation of mongooses, but Dammin and Wheller (1945) could not infect these animals

Kempf, Wheeler, and Nungester (1945) isolated Ann Arbor virus from hamsters inoculated with human throat washings It infects mice nasally, and antisera can be prepared in roosters It shares an antigenic component with mouse pneumonitis virus (St John and Gordon, 1947, see Table 32)

a size of 40mμ (if spherical). The principal hydrated particles of combined virus have a diameter of 140mμ.

The following are the chief properties of the 4 states in which PVM may occur.

State of Virus	*Infectivity for Mice*	*Hemagglutination Unheated*	*Heated*
Free infectious	+	+	+
Free noninfectious	o	+	+
Combined infectious	+	o	+
Combined noninfectious	o	o	+

4. The virus is neutralized by about 30 per cent. of normal human sera (Horsfall and Hahn, 1940), and by sera from many animals (Horsfall and Curnen, 1946 *a*, *b*). It appears that infection with this agent is widespread, and it seems reasonable to conclude it is an occasional cause of human pneumonia

5. Streptococcal cultures given nasally 2–3 days before, or 1–4 days after nasal inoculation of virus in mice resulted in marked inhibition of virus multiplication in the lungs. Further experiments showed that the active substance was probably polysaccharide. Then it was found that certain other polysaccharides of bacteria (especially Friedlander's bacillus), and of nonbacterial origin were also capable of inhibiting the multiplication of virus in the lungs of mice The effect may be due to competition with PVM for some essential growth system (Horsfall and McCarty, 1947).

6. Tubercular lesions of the lungs in mice were enhanced if the animals were infected with PVM or influenza virus as well (Volkert *et al*, 1947).

The SF Virus

The San Francisco (SF) virus was isolated in 1940 from the lungs of 2 fatal and the sputum of 2 nonfatal cases of atypical pneumonia, and has been isolated from other cases (Eaton, Beck, and Pearson, 1941, Beck and Eaton, 1942; Meiklejohn, Beck, and Eaton, 1944).

1. Electron micrographs show the particles to measure 422 ± 58mμ (Kurotchkin *et al.*, 1947).

2. It infects mice nasally and cerebrally, but does not kill by the peritoneal route Cotton rats, hamsters, and guinea-pigs can be infected (see Table 31). It is of low virulence for Java rice birds The virus does not produce the carrier state in mice or birds. It grows in the allantoic cavity (Francis and Gordon, 1945).

3. The virus is antigenically related to other members of the group, but occupies a fairly distinctive position (see above).

The Illinois Virus

Zichis and Shaughnessy (1945) isolated a pneumonitis virus from the lungs of 2 fatal cases of pneumonia by mouse inoculation. It was related to but distinct from SF. It was different from psittacosis, ornithosis, or MP.

The Louisiana Virus

This virus was isolated from an outbreak of AP in the Bayou region of Louisiana (Binford and Hauser, 1944, Olson and Larson, 1944, 1945, Olson and Treuting, 1944, Treuting and Olson, 1944, Fite, Larson, and Olson, 1946, Larson and Olson, 1946).

1. Autopsy of human cases showed pulmonary consolidation, infiltration of the alveoli with large mononuclear cells, hyperplasia of the alveolar lining cells, with at most slight involvement of the bronchioles and bronchi A few basophilic cytoplasmic inclusions were found in the pulmonary alveoli

2. The virus is readily transmissible to mice, guinea-pigs, and cotton rats, by the peritoneal route White rats, hamsters, and deer mice are not so susceptible

Physical examination may detect moist sounds, dullness, and altered breath sounds in the lungs, pharyngitis, the temperature usually varies between 100° F. and 103° F for 7–10 days, and falls by lysis.

Atypical pneumonia may occur in association with malaria (Campbell, 1943; Applebaum and Shrager, 1944, Fleming, Lindeck, and Evans, 1945). Complications include pleural effusion and skin rashes Bronchiectasis has been reported (Blades and Dugan, 1943, Kay, 1945, Nalls, 1945, Schmitz, 1945), also pericarditis (Finkelstein and Klainer, 1944). Nervous complications may occur, such as polyneuritis, serous meningitis, meningomyelitis, and encephalitis (Campbell *et al*, 1943, Sheppe *et al.*, 1943; Noran and Baker, 1946, Holmes, 1947).

The fatality rate is not over 0.1 per cent, although it may be more severe (Golden, 1944, Higley, Warren, and Harrison, 1944)

CLINICAL PATHOLOGY

There is not usually any significant change in the total or differential white count, but there may be a relative increase in mononuclears. Some workers have described an increase in the BSR, but others have not found the test of value (Willcox, 1943, van Ravenswaay *et al*, 1944, *Commission*, 1946, Painton, Hicks, and Hantman, 1946) The cephalin-cholesterol flocculation test is said to be positive (Adams *et al*, 1946) Hypo-amino-acidemia does not occur (Emerson *et al.*, 1943).

The serum may develop a capacity to fix complement with various crude tissue antigens (Thomas *et al*, 1943 *a*), and heterophil antibodies for horse cells have been described (Adams *et al*, 1946)

"Cold" agglutinins develop in the sera of half to a third of all cases, and have been studied by many workers (see, e g, Dameshek, 1943, Hortsmann and Tatlock, 1943, Meiklejohn, 1943, Peterson, Ham, and Finland, 1943, Shone and Passmore, 1943, Turner, 1943, Turner and Jackson, 1943, Turner *et al*, 1943, *Commission*, 1944 *a*, 1946, Heintzelman and Seligmann, 1944, Humphrey, 1944, Rich, Rae, and McGoey, 1944, Siffert and Krautman, 1944, Streeter, Farmer, and Haynes, 1944; Eaton, 1945, Erf, 1945, Finland, Peterson, and Barnes, 1945, Finland *et al*, 1945, Florman and Weiss, 1945, McNeil, 1945, Meiklejohn, Eaton, and van Herick, 1945, Springarn and Jones, 1945, Abernethy, 1946, Hegglin, 1946, Young, 1946, Eaton and van Herick, 1947 *a*)

1 The hemagglutinin operates at about 0° C It is completely independent of the blood group isohemagglutinins. In diagnostic tests, human group o cells are used

2 Similar agglutinins may develop in other conditions, but titers of 1/64 or more are seldom found except in cases of AP, in general, the severer the illness the higher the titer The test first becomes positive 4–8 days after onset, and the maximal titer is usually reached between the 16th and 23rd days. A fourfold or greater increase is very rare except in AP (*Horsfall*, 1947) The test is usually negative after 4–6 weeks

3 The agglutinin is destroyed at 66° C in ½ an hour, and may be removed by filtration (Finland, Peterson, and Barnes, 1945).

4 There is some risk that under certain conditions an intravascular hemolytic reaction may occur, owing to agglutination and lysis of red cells (Dameshek, 1943, Helwig and Freis, 1943, Finland *et al*, 1945, Platt and Ward, 1945).

5 Cold agglutinins do not develop in all cases of apparently typical AP, and pending clarification of the etiology, Williams (1947) has suggested that a distinctive group can be recognized, characterized by patchy radiological shadows, migration of the pneumonic process from one lobe to another, and the presence of cold agglutinins to a titer of 1 64 or over

Streptococcal agglutinins The sera of up to half the cases of AP develop agglutinins for various "indifferent" streptococci, such as the MG or 344 strains (Thomas *et al*, 1943 *b*, Meiklejohn and Hanford, 1944, Finland, Samper, and Barnes, 1945, Eaton, 1945, Florman and Weiss, 1945, Abernethy, 1946, Eaton and van Herick,

CHAPTER LXIX

PRIMARY ATYPICAL PNEUMONIA (AP)

For many years, clinicians have from time to time described cases of consolidation of the lungs, *differing markedly from the classical lobar (pneumococcal)* pneumonias or from bronchopneumonias. These cases seem to have become more common in the last 10 years, and the subject has attracted much attention (see, e g, Reimann, 1941, 1943, Beeson, 1943, Drew, Samuel, and Ball, 1943, Favour, 1943, Smadel, 1943, Francis, 1944; Moore, Wightman, and Showacre, 1944; Dingle, 1945; Lembcke and Young, 1945, Curnen, 1946).

The etiology of nonbacterial pneumonia is obscure. It appears that in about 25 per cent. of cases, the causal agent is a basophilic virus of the psittacosis-ornithosis-pneumonitis group. Certain other viruses may produce a similar clinical picture, for example, *lymphocytic choriomeningitis (Reimann, Havens, and Price, 1942)*, and influenza. A similar form of pneumonia may be produced by *Rickettsiae*.

However, the bulk of cases is not due to these agents, and the cause awaits discovery. There is little doubt, particularly after the careful experiments of the *U S. Commission* (1946), that the causal agent of the common form of nonbacterial pneumonia is a filtrable virus.

There exists a group of milder infections of the upper respiratory tract, and some of these cases may well be caused by the same virus as produces nonbacterial pneumonia (see, e.g., Iverson, 1943, *Commission*, 1944 *b*, *c*)

As the etiology of mild nonbacterial pneumonia is uncertain, terminology cannot but be confusing Those cases produced by psittacosis and ornithosis virus should be described as psittacosis or ornithosis Cases produced by Louisiana, SF, or other viruses may be described as "virus pneumonias" produced by these agents Most cases, in which the causal agent is unknown, are usually now described as "primary atypical pneumonia" (AP), and the addition "etiology unknown" may be used to obviate confusion with other virus pneumonias The unqualified term "virus pneumonia" is too general to be of much value, and could include infection by the viruses of psittacosis, ornithosis, pneumonitis, influenza, and measles, as well as infection by the yet undiscovered virus of AP

We see no reason why the term "pneumonitis" should be used instead of the time-honored "pneumonia" Unfortunately, the term has come to stay as regards certain basophilic viruses of human and animal origin related to psittacosis.

CLINICAL FEATURES

The clinical features have been described by many different workers (e g, Brown *et al.*, 1942– [illegible] Engelhardt and Wilen, 1942, Goodrich and Bradford, Heyden, 1942, Riven and *Stern, 1942*, B 1943, *1944 a*, Drew, Samuel, and Ball, 1943, Haight and Trolinger, 1943, Meakins, 1943, Montgomery and Shore, 1943, Young, Storey, and Redmond, 1943, Favour, 1944, Fraser, 1944, Gundersen, 1944, Higley, Warren, and Harrison, 1944, Hobby, 1944, Sachs, 1944, Smith, 1944, Breslow, 1945, *Commission*, 1945 *a*, 1946, Curnen *et al*, 1945, Fuchs, 1945, Grossman, 1945, Karpel, Waggoner, and McCown, 1945, *Abernethy, 1946*, McDonald and Ehrenpreis, 1946)

The onset is usually gradual, with influenza-like or upper respiratory symptoms including chills, anorexia, and headache Local symptoms include nasal stuffiness, sore throat, dry paroxysmal cough, mucoid sputum, and pain in the chest or abdomen

1944 *c*, Erickson and van Ravenswaay, 1944, Owen, 1944, Tumulty, 1944, Glendy, Beaser, and Hankins, 1945, Turner, 1945; Abernethy, 1946, Adams *et al*, 1946).

EVIDENCE FOR A VIRUS ETIOLOGY OF AP

Clinically, the condition that we describe as primary atypical pneumonia is distinctive, and not to be confused with bacterial pneumonia. The x-ray changes may be regarded as more suggestive of a virus than a bacterial pneumonia No worker has succeeded in proving the rôle of any cultivable bacteria or other agent in the disease In fact, there seems little doubt that the condition is due to a filtrable virus. That this is so, has been proved by the elaborate experiments of the U S. *Commission* (1945 *b*, 1946, 1947; Dingle, 1945). This group showed that the disease can be transmitted to volunteers by sprays of filtered (and unfiltered) throat washings and sputa of typical cases The incubation period was found to be 8–14 days, being longer when filtrates were used The illness was typical of the naturally occurring disease Cold agglutinins developed in most cases, and streptococcal agglutinins in some instances

In a number of cases, a less severe form of minor respiratory illness developed This suggested that the same virus might cause a gradation of symptoms

As yet, no reports on the characters of the filtrable agent incriminated by the Commission have appeared

The Virus of Atypical Pneumonia

This virus has been isolated by Eaton and his collaborators, from sputum and lung suspensions of cases of AP (Eaton *et al.*, 1942, 1945; Eaton, Meiklejohn, and van Herick, 1944; Eaton 1945, Eaton, van Herick, and Meiklejohn, 1945; Eaton and van Herick, 1947 *a*, *b*).

The virus is transmissible to cotton rats, hamsters, and chick embryos, the particle diameter lies between 180–250 mμ

It has proved possible to immunize hamsters and cotton rats by injections of live or formolized virus.

This virus would seem to be of definite importance, as specific neutralizing antibodies have been found in the convalescent sera of a considerable number of cases in different outbreaks of clinically typical AP in both eastern and Pacific parts of America These sera have also contained cold and streptococcal agglutinins

PNEUMONITIS IN INFANTS

Adams and coworkers described a variety of primary pneumonia occurring in infant nurseries (Adams, 1941 *a*, *b*, 1943, Adams *et al*, 1942) The infection was highly contagious, and affected the very young. The incubation was about 7 days The affected babies had a cough, and showed dyspnea and cyanosis There was a low pyrexia About 25 per cent died of hemorrhagic pneumonia Cytoplasmic inclusions were found in the bronchial epithelial cells Ingleby (1944) found similar inclusions in various organs

Goodpasture *et al* (1939) described nuclear inclusions in the tracheal and bronchial epithelium of infants

No definite viruses were, however, isolated by the above workers.

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1947 *a*). Agglutinins for these streptococci develop more commonly and to higher titer in the sera of cases of AP than in other conditions. The streptococcal agglutinin is unrelated to the cold hemagglutinin. A fourfold or greater increase is only rarely found except in AP (Horsfall, 1947).

Although nonhemolytic streptococci can be isolated from the respiratory tract of cases of AP, there is no evidence that any particular serological group is causally associated with the disease (Dingle *et al.*, 1944 *b*; Thomas *et al*, 1945); the significance of the presence of these agglutinins is, therefore, obscure.

Laboratory diagnosis. Some of the laboratory tests required will be evident from what has be[illegible]
be refrigera[illegible]
to isolate a [illegible]
and by inoculation of yolk sacs, complement fixation tests with psittacosis or LGI antigens, and perhaps the Frei test will help to exclude those cases due to basophilic viruses. Ziegler *et al* (1947) have pointed out that for the accurate diagnosis of AP, a very full series of laboratory tests is required, including tests for influenzal infection, for pneumococci, for streptolysin, as well as the tests mentioned above. Using such a system to investigate a group of cases of respiratory infection, they found that AP and influenza might occur together, and that cases classified clinically as AP often turned out to be pneumococcal in origin.

RADIOLOGY

The appearances, although not diagnostic, are most helpful, and certain cases can be diagnosed only by this means (Fredd, 1941, Duggan and Powers, 1942, Campbell *et al*, 1943, Correll and Cowan, 1943, Haines and Forcey, 1944, Kruger *et al*, 1944, Showacre, Wightman, and Moore, 1944, Yoskalka, 1944, Abernethy, 1946, Crysler, 1946, McDonald and Ehrenpreis, 1946).

The changes usually occur unilaterally, and various appearances have been described a wedge or fan-shaped infiltration in the lower lobe, with its apex at the hilus; patches of irregular opacities, and even up to miliary lesions, peribronchial infiltrations, reticulation radiating from the hilus, apical lesions may occur resembling those of tuberculosis. Changes suggestive of peribronchial fibrosis may persist for several months X-ray therapy has been claimed to be effective (Correll and Cowan, 1943)

PATHOLOGY

Comparatively few reports of pathological investigations have appeared (Campbell *et al*, 1943, Perrone and Wright, 1943, Golden, 1944, Lusk and Lewis, 1944) The lesions are essentially those of an acute interstitial pneumonia The chief changes include bronchitis, bronchiolitis, peribronchitis, and peribronchiolitis Mononuclear infiltration is common Atelectasis is caused by a mechanical obstruction of the bronchi and bronchioles Microscopically, the alveoli contain mononuclear and polymorph cells, with fibrin A secondary bacterial invasion may occur

EPIDEMIOLOGY

The disease seems to affect chiefly young adults, mainly males It occurs in all parts of the world, including the tropics (see, e g, Bower, 1943, Leake and Blatchford, 1943) It has been suggested that there are seasonal peaks of high incidence in November or March.

The disease is less common in "seasoned" troops than in new recruits (*Commission*, 1944 *b*). During the second World War, AP was responsible for a considerable temporary loss of manpower, and in this respect was probably more important than bacterial pneumonia (Markham, 1942, Moore, Tannenbaum, and Smaha, 1942, Campbell *et al*, 1943, Drew, Samuel, and Ball, 1943, Frachtman, 1943, *Commission*,

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1909	Zulpich	Finkler (1910), Bichem, Selter, and Finkler (1910)
1914	England	Beddoes (1914)
1924	Edinburgh	Gulland (1924).
1927	London	Stolkind (1927).
1928	New York.	Sailer (1928–9)

Some Later Outbreaks

South America	Argentine	Barros (1929, 1930, 1940), Sibate (1929), Marquez (1929), Pattin (1929), Bozzola *et al* (1937), Loizaga and Averbach (1944), Vilches and Averbach (1944)
North America	Philadelphia	Sailer (1928–29)
	New York	Rabinowitz and Livingstone (1932)
	Maryland	Ellicott and Halliday (1930)
	Ohio	Haines (1930)
	Pennsylvania	Bortz and Green (1930)
	New Orleans	Wirth (1930)
	Washington	Peterson, Spalding, and Wildman (1930), *Public Health Reports* (1938)
	Massachusetts	Wellman (1930)
	Pittsburgh	Maclachlan, Permar, and Rogers (1930)
	"	Hoge (1934 *b*), Badger (1934)
	Canada	McIntosh (1932), Prendergast and Phair (1941), Teney (1941)
Japan	Yokohama	See *J Amer med Ass* (1930), 95, 1360
Germany	Hamburg	Hegler (1930), Wohlwill (1930), Gräff (1930), Adimy (1930)
	Altona	Gunther (1930), Lmbden and Adimy (1930)
	Cologne	Pesch and Siegmund (1930)
	Horst	Kaliebe (1930)
	Dortmund	Lons (1930)
	Berlin	Elkeles (1930 *a*), Grunwald and Meyer (1930), Ehrmann (1930), Haagen and Mauer (1939), Tornack (1941 *a*)
	Munich	Kerschensteiner (1930)
	Mulheim	Beyreis (1936)
	Dresden, Glauchau, Doebbeln (Saxony), Chemnitz, Hartha, and Munster	Roubakine (1930)
	Wiesbaden	Koch (1940)
Austria	Vienna	Weltmann (1929), Gerlach (1936)
	Graz	Widowitz (1930)
Czechoslovakia	Prague	
	Moravska-Ostrava	Roubakine (1930)
Netherlands		Herderschêe (1930), Ruys *et al* (1939), Minkenhof, Ruys and Vervoort (1938)
Switzerland	Geneva	Roch and Wohlers (1930)
France	Paris	Sacquepee and Jame (1930), Aujaleu and Jude (1936)
Spain and Portugal		Roubakine (1930)
Britain		Thomson and Hillier (1930), Hutchinson *et al* (1930), Bedson, Western, and Simpson (1930), Horder and Gow (1930), Sturdee and Scott (1930), Morgan (1938), McMillan (1940)
Australia	Victoria	Burnet and Macnamara (1936), Kennedy (1936)
	Adelaide	Yeatman and McEwin (1945)

CHAPTER LXX

PSITTACOSIS

Introduction

JUERGENSEN (1874)[1] is credited with being the first to recognize the disease psittacosis as a distinct clinical entity manifesting itself as an atypical form of pneumonia. Later it was referred to as "pneumotyphus" by Ritter (1880) and Wagner (1884, 1888).

It occurred in Paris in 1892, and Morange (1895), who studied cases in France, proposed the name of "psittacosis" for the malady. At this time Nocard (1893) claimed to have discovered the cause of the condition, and isolated a bacillus from fatal cases of the illness

It was not until the 1929–30 outbreak that Lillie (1930) in America, the British microscopist Coles (1930), and Levinthal (1930 *a*) in Germany discovered almost simultaneously the presence of minute intracellular bodies within affected reticulo-endothelial cells. Subsequent researches of Bedson (1930), Gordon (1930), and Bedson and Bland (1932) firmly established the etiological relationship of these structures to the malady, and further revealed that the virus exhibited a cycle of morphological changes when growing in animal tissues. Owing to its general resemblance to the *Rickettsiae*, Lillie (1930) has called the parasite *Rickettsia psittaci*, and Coles (1930) likewise stressed its resemblance to the *Rickettsiae*. Such a designation is not desirable, however, for it implies that the agent is associated at one stage of its life history with an arthropod vector, and this has not been proved.

The term *Mycobacterium multiforme psittacosis* proposed for it by Levinthal (1930 *c*) is also unsatisfactory.

In the following pages we propose to refer to the agent as the virus of psittacosis; we have also adopted the name of Levinthal-Coles-Lillie body, or LCL body, to describe the inclusion body stage of the virus

For information regarding the general aspects of this disease the reader is referred to the work of Bedso [illegible] (1932), Meyer, Eddie, and Stevens (1935), Rivers [illegible] (1937), Meyer (1937, 1942), Cardona (1937), Bar [illegible] (1944)

Psittacosis is one of the varieties of virus pneumonia It has been estimated that about 25 per cent of cases of sporadic atypical pneumonia are due to psittacosis (Smadel, 1943) The antigenic relationships in the LGI-psittacosis-pneumonitis group are described in Chapter LXVII.

EPIDEMIOLOGY

Some Early Outbreaks of Psittacosis (before 1929)

1879	Switzerland	Ritter (1880), Eberth (1880)
1882	Leipzig	Ost (1883), Wolff (1883)
1882	Bonn	Finkler (1888)
1892	Paris	Gastou (1892), Morange (1895), Du Jardin-Beaumetz (1892)
1895	Florence.	Palamidessi (1895)
1895	Prato and Genoa	Malenchini (1896)
1896	Paris	Dupuy (1897), Gilbert and Fournier (1896), Nocard and Debove (1896), Sicard (1897)
1897	Genoa	Maragliano (1897)
1898	Stettin	Haedke (1898).
1904	Brazil	Souza (1904)

[1] References are appended at the conclusion of Ch LXXI, p 758 et seq

isolated, but in some of them, although the spleen was not pathogenic to mice, the organ was found to be greatly enlarged and this was accordingly interpreted as a sign of past infection The virus has also been isolated from Australian shell parakeets by Meyer and Eddie (1934), who found that freshly isolated strains were at first feebly pathogenic to mice, but after repeated passage they could be exalted in virulence, and typical LCL bodies were then demonstrable in the spleens of infected animals. Two strains of virus isolated from Australian parrots of the species *Psephotus haematonotus* and *Platycercus eximmo* have been successfully cultivated in the chorio-allantoic membrane of the developing chicken embryo by Burnet and Rountree (1935).

Later, Burnet (1939) isolated virus from wild parrots in Victoria, Tasmania, and S Australia (see also Meyer and Eddie, 1939 *b*). Finches may be infected (Tremain, 1938). Geiger *et al.* (1939) found the following infected *Alisterus*, *Platycercus*, *Nymphicus hollandicus*, *Kakatoe sanguinea*, and *Kakatoe galerita*.

Conclusions: Species of Bird Affected

At least 50 species, comprising 5 orders of the class *Aves* may be infected spontaneously with psittacosis (Meyer, 1942). The bulk of infection seems to be in Australia and S America So far, Indian parrots appear to be free of infection (Goyal, 1940)

The Mode of Spread of Psittacosis

During the last epidemic of the disease, which occurred in 1929–30, a number of important points were reestablished concerning the epidemiology of psittacosis, the most outstanding of which was the fact that the appearance of the human disease was preceded by an epizootic among parrots, probably in Brazil (see Roubakine, 1930). Many of the birds which were imported by dealers into the Argentine, Great Britain, and the Continent died during transit, or else shortly after arrival at their destination Weltmann (1929), for example, states that out of a consignment of 157 Brazilian parrots which arrived at Bremen during the end of July 1929, only 40 survived the journey to Vienna. Thirty of these were bought by an American and the remaining 10 by other persons, in each of whose households cases of psittacosis subsequently developed. A somewhat similar state of affairs was experienced in the Paris outbreak of 1892, when 2 bird dealers purchased 500 parrots from Buenos Aires and only 200 of the creatures were delivered alive in Paris

At one time the facilities offered on board ship for the conveyance of birds were extremely insanitary (see Wolff, 1883, Pitman, 1927, Bannerman, 1927, and Heymann, 1930), but even although livestock transport arrangements are much improved to-day, a great number of birds perish *en route*

Adverse conditions, such as chilling and wetting, act by causing the disease to become active in birds latently infected Such carriers become sick and excrete virus, *infecting others previously healthy*

Spread of infection from parrot to parrot.

Theoretically any object which has been in contact with an infected parrot is potentially capable of infecting another bird. Thus, healthy stock are liable to catch the disease if placed in infected cages [illegible] saliva, droppings, or urine of sick parro[illegible] have demonstrated the presence of the [illegible] also possible that the virus may sprea[illegible] surroundings or in captivity, as the result of direct mouth-to-mouth contact.

Spread of infection from parrot to man.

Probably the majority of human cases of psittacosis has been contracted by bird owners and others who touch the cages or handle the plumage of infected

Species of Birds known to Transmit Infection

In his survey of the literature on human *psittacosis*, Roubakine (*1930*) stated that up to that time nearly all the described cases of psittacosis had been caused by parrots originating from the Amazon district of Brazil. Most of the cases were c[illegible] *cus*, known as the "[illegible] was the home of t[illegible] id disease among birds transported from the tropics. He stated that because in the 1929-30 epidemic the condition appeared synchronously in Argentina, Algeria, Germany, Austria, England, Holland, Czechoslovakia, Denmark, Switzerland, France, Spain, Portugal, Canada, Hawaiian Islands, and the United States, he considered it improbable that so world-wide a distribution could spring from a single country of origin. In view of these conflicting opinions, the origin of the 1929–30 outbreak cannot be definitely located. The bulk of evidence is in favor of South American birds being the source of infection, and that the 1929–30 cases in man were preceded by an epizootic among parrots in Brazil At this time psittacosis was an unknown disease in Australia, consequently birds imported from this continent were exempt from suspicion (*vide infra*) (see Elkeles and Barros, 1931).

Parakeets, "love birds," and canaries have also been found infected (Meyer and Eddie, 1933 *a*, Rosenbusch, 1937). Thus the Zulpich epidemic of 1909 was due to the grass parakeet *Melopsittacus undulatus;* thrushes and the Italian cardinal bird have been blamed by Barros (1929). Budgerigars have been incriminated by Swan and Dawson-Walker (1939) and others.

Forest psittacines (*Myiopsitta monacha* Bodde) have been found infected in South America (Parodi and Silvetti, 1946).

Psittacosis in Australia

At one time it was believed that the disease was absent in Australia, but later Meyer and Eddie (1934), in California succeeded in isolating the virus from a consignment of 200 Australian shell parakeets which they had imported from Sydney. Investigations were next set on foot to discover whether the disease existed among Australian birds living amid their natural surroundings, as a result of which Burnet (1934) succeeded in recovering the virus from a number of birds purchased in Adelaide and Melbourne No human cases were, however, observed at the time, and in an article dealing with this subject in *Health* (1936) it was pointed out that the apparent absence of human cases in Australia was probably attributable to the fact that, since the strains of virus isolated from Australian parrots were found to be feebly pathogenic to mice, they were, in all probability, also equally avirulent to man. Following these observations, a careful watch was kept for the occurrence of human cases in Australia, and Kennedy (1936) demonstrated the virus in the sputum of one patient suffering from the disease Ross and Rabinov (1936) subsequently reported 7 cases of infection acquired through contact with sulfur-crested cockatoos of the species *Cacatua galerita*, and Burnet and Macnamara (1936) stated that there were approximately 17 human cases in the State of Victoria, Australia, from 1935 to 1936 In 2 instances the disease was traced to contact with aviary-bred budgerigars of the species *Melopsittacus undulatus*, and in the remainder to sulfur-crested cockatoos (see also Merrillees, 1934, and Cumpston, 1936)

Burnet (1935) has proved the presence of latent psittacosis infection in the spleens of several of the commoner species of Australian parrot The virus could be recovered from these birds by removing the spleen, emulsifying it in broth, injecting the material into mice and thereafter examining the spleens of the latter for evidence of LCL bodies. By this method Burnet succeeded in isolating strains of psittacosis virus from the *Psephotus haematonotus*, *Trichoglossus*, and *Leptolophus hollandicus* varieties of parrot From other species the virus was less frequently

to mice, and it is conceivable that these may be able to infect human beings and particularly laboratory workers who fail to take adequate precautions to safeguard themselves against infection (see McCoy, 1934).

Quarantine Measures

To obviate the risk arising from the importation of infected stocks of birds, quarantine of up to 6 months should be imposed on all shipments of psittacines. During this period, the infected animals usually become sick. This quarantine procedure is abundantly justified. For example, in a shipment of over 100 Amazon parrots and parakeets quarantined in Texas, no less than 97 per cent. died (Dunnahoo and Hampton, 1945).

Geiger *et al.* (1939) draws attention to the following points

1. Clinical examination alone is useless to detect infection
2. Laboratory examination is specially indicated if deaths have occurred in transit.
3. Enlargement of the spleen is suggestive of infection
4. The highly susceptible rice bird (*Padda oryzivora*) should be distributed in cages about the quarantine house, to act as an indicator of "shedding" of virus.

A useful practice is to examine a definite proportion of the shipment by autopsy, and carry out virus isolations and serological tests

CLINICAL FEATURES OF PSITTACOSIS

The *incubation period* ranges from 6 to 15 days, and is generally 10 days in the majority of cases, but sometimes may be as long as 30 to 82 days (see Gulland, 1924, Armstrong, 1930).

Onset is often sudden, and the illness may be ushered in by a chill, sore throat, headache, or vomiting, followed by signs of general malaise, such as insomnia and delirium. Either diarrhea or constipation may be evident at this stage In the case of individuals who have contracted infection as the result of feeding the animals with their mouths, circumoral edema has been noticed Other clinical manifestations are furring of the tongue, loss of appetite, abdominal distension, epistaxis, or stupor

Sex Females are more frequently affected than males, but this difference is probably attributable to the fact that women are more often exposed to infection than men For example, during the course of their domestic duties they spend a good deal of time at home, in the proximity of infected birds, and are particularly liable to acquire infection during the act of feeding the animals or cleaning out their cages

Age is an important factor in determining the prognosis of a case Armstrong (1930) stresses the fact that children and young adults suffer from milder attacks of the disease than their elders, and in a series of 169 cases investigated by him he found that no deaths occurred among 35 persons under 30 years of age, whereas the mortality rose to 24 per cent in individuals over that age Thomson and Hillier (1930) have also commented upon the resistance of young children to infection and state that when they do develop the disease they experience less severe symptoms than adults

Symptomatology

Lung lesions.

Pulmonary changes are a characteristic feature of human psittacosis, but they may or may not be detectable at the onset of the illness, and their existence is not necessarily a bad prognostic sign At first the patient's signs and symptoms resemble those of a mild attack of bronchitis, and the clinician may diagnose the case as one of early lobar pneumonia Later, however, about the end of the first 7 days, various points of distinction become apparent, and the absence of a malar flush, persistent

birds, such as is unavoidable when cleaning out cages or perches. In other cases there may be no record of the individual having handled the bird himself, and in such instances infection has been traced to respiratory droplet infection, due to the person having been near to the animal when it was speaking or screeching Lastly, infection has been acquired by certain bird fanciers who practice the objectionable habit of feeding their pets directly out of their own mouths. To do so with an infected bird is obviously to invite disaster. The feathers and wings of dead birds can convey the virus to taxidermists, milliners, and others.

It must be emphasized that birds may not appear to be ill at all, but may be carriers of the infection and "shedding" virus

Infected material, in the form of dried particles, is abundant in the feathers of birds The risk to man is by inhalation of such particles, or droplets of respiratory secretion.

Psittacosis has occurred in the staffs of zoological gardens, and such workers should wear masks and goggles (Troup, Adam, and Bedson, 1939, Tomlinson, 1941).

Spread of infection from man to man.

Man-to-man infection sometimes occurs, but is a comparatively rare event Cases of disease acquired by those in contact with patients such as nurses, doctors, visitors, and others have been reported in the literature by Armstrong (1930) in America, by Bedson, Western, and Simpson (1930) in England, and by Gunther (1930) and Hegler (1930) in Germany. Lillie (1933) demonstrated EB's in the alveolar secretion, accordingly, the case must be treated as infectious (see Pfaffenberg, 1936).

Human carriers of psittacosis virus.

Gerlach (1936 *b*) has described 5 cases of latent infection in whom the classical signs and symptoms of the disease were absent. In none of these individuals could the infection be traced to contact with birds In 2 instances there was a history of contact with other cases of psittacosis in hospital, and in 2 others the virus was discovered fortuitously during the conduct of a search for carriers among a group of 50 unselected patients On one occasion the virus isolated was so feebly pathogenic to mice that it was necessary to passage it serially through 6 sets of animals over a period of 6 weeks before it produced recognizable inclusion and elementary bodies in the spleen. In another case the agent was recovered from the patient's blood on the 72nd day, and from the sputum on the 75th day after the diagnosis had been originally established Gerlach's findings are of considerable interest, since they illustrate the important fact that human psittacosis is not invariably accompanied by recognizable clinical signs and symptoms or evidence of a severe illness The work, furthermore, emphasizes the importance of regarding all individuals engaged in the handling of parrots, parakeets, and other varieties of exotic bird as possible carriers of infection The necessity for repeated passage of the virus in mice before recognizable lesions are produced in the animals is also made clear

Laboratory infections.

These infections have been described An interesting case was that of Rosebury *et al* (1947 *a*) where the source was a faulty ampule More usually infection is contracted during the manipulation of suspensions or the inoculation of animals or eggs

The Rôle of Other Animals in the Spread of Psittacosis

Monkeys are susceptible to experimental inoculation with the virus, and it is quite possible that in captivity they may become infected from birds, and then convey infection to human beings (see Lons, 1930) The virus is also pathogenic

publications of Kerschensteiner (1930), Lons (1930), Rivers, Benjamin, and Berry (1930), and Johnson and Levinthal (1935).

Pulse and respiration rate are usually increased, and the rise may be considerable in severe cases

Relapses sometimes occur. In the 11 cases reported by McCoy (1930) affecting laboratory workers, 3 suffered from relapses within 3 weeks after their temperature had subsided to normal, and another patient experienced a relapse after 4 weeks. All 4 relapses occurred among patients who had been treated with convalescent serum during their illness

Complications Thrombophlebitis has been known to occur, and a common cause of death appears to be pulmonary thrombosis (Armstrong, 1930, Rabinowitz and Livingstone, 1932; Badger, 1934).

MORTALITY

The mortality figures reported by independent observers during different epidemics of psittacosis have fluctuated considerably Dupuy (1897) stated the death rate to be 34 per cent. among 70 cases observed by him over a period of 4 years in Paris Armstrong (1930) examined the case records of 169 patients during the American outbreak of 1929–30, and found the mortality to be 19 per cent, and Nocht found the figure to be 35 per cent among 20 cases of psittacosis observed by him in Hamburg Altogether from July 1929 to February 1930, Heymann (1930) estimated that a total of 350 to 400 cases was notified throughout America and Europe and of these 35 to 40 per cent. died

All races of mankind appear to be equally susceptible to psittacosis infection, and there are no reasons to believe that different individuals possess either racial or natural immunity to infection It is nevertheless a curious fact that although the disease is enzootic among parrots in Brazil, fewer human cases appear to have been described by clinicians from this part of the world than elsewhere (see Souza, 1904, Roubakine, 1930)

CLINICAL PATHOLOGY

The leukocyte count in psittacosis has been investigated by Krumeich (1930) and his results show that the white count is usually normal at the beginning of the illness, but later a transient leukocytosis may occur, followed by a leukopenia. Sturdee and Scott (1930) maintain that the leukocyte count is normal or subnormal in 75 per cent of cases, and that leukopenia is present in only 25 per cent of the patients In the cases studied by Armstrong (1930) the white count fell to 3,000–5,000 cells, and in one case a reading of 500 leukocytes per c mm was observed on the 20th day of the patient's illness (see also Rivers, Benjamin, and Berry, 1930, Thomson and Hillier, 1930, and Peterson, Spalding, and Wildman, 1930) The red cell count sometimes shows a slight fall at the height of the febrile period, but is usually within normal limits.

Albuminuria is commonly present during the febrile period

PATHOLOGY

The Respiratory System

The lungs.

The postmortem appearances naturally depend on the stage of the illness at which death occurs If it takes place on or about the 13th day of the illness, then the lungs are congested, and gray or grayish-red consolidation may be seen. The *pleurae* are smooth, they exhibit petechial hemorrhages, and a turbid serofibrinous exudate may be present (see Horder and Gow, 1930, Scholte, 1930, Krumeich, 1930)

The *nature of the consolidation* found in parrot disease has been carefully in-

coughing, and profuse expectoration enable the condition to be differentiated fror typical lobar pneumonia.

Physical examination of the chest On inspection, seen after the first week, ther may be unilateral limitation of thoracic movement. On palpation and percussio there may be pain, and other signs of pulmonary involvement can be elicited. Th earliest demonstrable changes may be confined to an area of dullness on percussio at one or other base, which may spread to involve other parts of the same organ or extend to the opposite side.

On auscultation over the affected area normal breath sounds are replaced b bronchial or tubular breathing, and this is soon followed by complete absence o all sounds and indications of generalized pulmonary consolidation Crepitations ar usually heard at the base and moist râles may be audible in other areas surroundin the focus of damaged lung (see Gulland, 1924; Thomson and Hillier, 1930, Kummerling, 1937). Gross-Hardt (1935) has given an account of the clinical finding and diagnosis of 7 cases.

In general, the lung lesions found in psittacosis tend to be patchy in their distribution, and from the results of physical examination they present the characteristics of multiple bronchopneumonic areas of consolidation, rather than the physical signs of a true lobar pneumonia. In cases in which recovery occurs, the pulmonary lesions may last throughout the course of the patient's febrile period and may even persist for some time afterward during convalescence

Pleural effusions have been reported by certain observers. Gulland (1924) removed about 10 oz. from a case studied by him, and found that it contained no cultivable bacteria. Thomson and Hillier also noticed pleuritic effusions in certain of the cases under their care. Rabinowitz and Livingstone (1932), on the contrary, remarked that even although extensive pulmonary consolidation was present in the cases studied by them, no evidence of pleural involvement could be elicited, and Maclachlan, Permar, and Rogers (1930) reported similar findings

X-ray examination of the chest has been carried out by Peterson, Spalding, and Wildman (1930), who have demonstrated the presence of pulmonary consolidation during life by radiographic methods (see also Rabinowitz and Livingstone, 1932, and Stehr, 1935).

Other signs and symptoms.

The skin. Horder and Gow (1930) reported the presence of a rash in one of the cases under their observation A colored illustration depicting the nature of this rash is to be found in the work of Sturdee and Scott (1930)

Central nervous system Several clinicians have reported the presence of headache, drowsiness, apathy, and mental depression during the course, and immediately following an attack of psittacosis (see Gulland, 1924, Rabinowitz and Livingstone, 1932).

Alimentary system The tongue is furred, being white at the onset and brown later during the disease, the throat is congested Either constipation or diarrhea may be present Physical examination of the abdomen may reveal no characteristic findings, but Horder and Gow (1930) reported the presence of abdominal distension about the 3rd week of illness.

Temperature There is a certain amount of variation seen in the temperature chart in psittacosis, and in a mild case of the disease it may rise gradually in steplike fashion, to attain a maximum about the end of the first week, and thereafter decline to normal In severe cases it usually tends to be high from the beginning, and may reach 103° or 104° F, and persist at that level with minor fluctuations for several days until death occurs

In general, it may be said that the appearance of the temperature chart in psittacosis conforms to the high and continuous type, and when recovery occurs it falls gradually by a process of lysis Other temperature charts are to be found in the

mitral, aortic, and tricuspid valves (see Reimann and Hutchinson, 1930, Hutchinson, Rowlands, and Simpson, 1930, Polayes and Lederer, 1932).

Vessels Thrombi have been frequently reported by different observers in the following sites pulmonary artery, right ventricle, the iliac and saphenous veins.

The Spleen

The spleen is slightly enlarged, it is soft in consistency and congested in appearance Microscopically the organ is markedly congested, infiltrated with lymphocytes and large mononuclear cells, and areas of hemorrhage, deposits of hemosiderin, and evidence of erythrophagia are also to be seen. Clumps of elementary bodies appear within [illegible] splenic sinuses (see Siegmund, 1930; [illegible] 1930, Polayes and Lederer, 1932, Ru[illegible]

Other Organs

Lymph glands The mediastinal lymph glands, and in particular those of the peribronchial group, are liable to be enlarged and swollen. On section they may contain minute hemorrhages and show reticulo-endothelial hyperplasia, with large lymphocyte and plasma cell infiltration, and areas of necrosis (see Russell, 1931; and Giese, 1930).

Bone marrow. In Haswell Wilson's (1930) case the femur marrow was practically depleted of polymorphonuclear leukocytes, and there was no evidence of proliferation of the myelocytes.

Muscles. Hemorrhages into the rectus abdominis and gluteal muscles as well as waxy degeneration have been observed (see Hegler, 1930, and Hutchinson, Rowlands, and Simpson, 1930).

Skin. Hemorrhages and ecchymoses into the skin are uncommon in psittacosis, but they have been reported by Ritter (1880) and Gastou (1892), and have also been recorded during life by Horder and Gow (1930).

Suprarenal glands Polayes and Lederer (1932) found that although the organ appeared to be normal when examined macroscopically, yet histological examination revealed the presence of congestion, lymphocytic infiltration, and parenchymatous degeneration of the medulla (Peterson, Spalding, and Wildman, 1930).

Kidneys There is congestion of the organ together with varying degrees of degenerative change involving the parenchyma

Esophagus, stomach, and *intestines* are often congested in appearance and minute hemorrhages have been described in their substance, together with infiltration by plasma cells and large mononuclear leukocytes The lymphoid follicles and Peyer's patches are occasionally enlarged slightly (see Polayes and Lederer, 1932, Hutchinson, Rowlands, and Simpson, 1930, Oberndorfer, 1930, Gunther, 1930)

Meninges, brain, and *spinal cord* Congestion of the meninges and infiltration with macrophage cells occurs In the brain, the tissue is hyperemic and multiple punctate hemorrhages appear around blood vessels in the *region of the* corpus callosum, putamen, internal capsule, thalamus, mesencephalon, aqueduct, and the floor of the 4th ventric[illegible]
ing in the claustrum, [illegible]
bellum and cerebrum [illegible]
Rowlands, and Simps[illegible]
1930, Hegler, 1930)

Sprunt and Berry (1936) have described the pathological appearances in a fatal case of psittacosis in a man aged 31 years. In the brain they found no evidence of swelling of neurons, neuronophagia, tigrolysis, or proliferation of glial cells, but there was slight increase in the amount of fat normally present in the ganglion cells Petechial hemorrhages occurred round the smaller blood vessels, and there were no indications of swelling, or degenerative or proliferative changes in the

vestigated by Peterson *et al* (1930) and also by Lillie (1933), who made an histological study of a number of different specimens derived from fatal cases of the disease. From his observations it was found that, although an affected lung might appear to be uniformly consolidated throughout when examined by the naked eye, the consolidation was really lobular in character and not evenly distributed throughout the tissue Thus, histological sections revealed that groups of either serum- or air-containing alveoli still remained in grossly consolidated portions of lung substance Another remarkable feature of the condition was the variability exhibited in the character of the cellular contents present in different alveoli Some of these were filled with serum, while others in addition to serum contained red cells, leukocytes, alveolar epithelium, and macrophage cells, present either individually or collectively with or without the existence of fibrin Lymphocytes and plasma cells also entered into the picture, but polymorphonuclear leukocytes appeared to be localized in the lumina of terminal bronchioles and atria. Many of the cells found in the exudate showed fatty degeneration, the alveolar epithelium and septa were edematous, and infiltrated with lymphocytes, macrophages, and a small number of polymorphonuclear leukocytes

The epithelium of the bronchial mucosa was almost completely desquamated, the medial coat of arteries became thickened, and capillaries were plugged with hyaline thrombi (Wilson, 1930, Polayes and Lederer, 1932; Mansens, 1934).

The pharynx, larynx, and trachea.

Polayes and Lederer (1932) have described the state of these organs in an autopsy performed by them Seen by the naked eye there was submucous edema and swelling of the larynx, trachea, and bronchi, together with hemorrhages beneath the surface of the epiglottis *Microscopically* the exudate in these regions consisted of mononuclear cells, plasma cells, neutrophil leukocytes and areas of extravasated blood The alveolar lining epithelium was desquamated, the cells were swollen, their nuclei enlarged, and numerous large mononuclear cells occurred In thin sections stained by Giemsa's method, clumps of minute purple-staining elementary bodies appeared as intracytoplasmic basophilic inclusion bodies within the alveolar cells, and Lillie (1933), who refers to them as *Rickettsia psittaci*, states that their demonstration can be facilitated in stained preparations by fixing the material in Mallory's permanganate oxalic acid fixative, instead of in formalin or Orth's solution

The Liver

This may be unaffected but sometimes it is slightly enlarged, congested, and areas of fatty degeneration are found on naked-eye examination *Microscopically*, sections show edema and swelling of liver cells with zones of paracentral and centrolobular focal necrosis The outline of the affected cells becomes shrunken, the nuclei are fragmented, their staining reaction tends to be oxyphilic in character, and tiny droplets of fat make their appearance inside the cytoplasm The intervening capillary spaces are filled with polymorphonuclear leukocytes, and sometimes hyaline thrombi The Kupffer cells are swollen, they contain phagocytosed erythrocytes, and more rarely LCL bodies are to be found within their cytoplasm

For detailed descriptions concerning the changes found in the liver in psittacosis the reader is referred to the publications of Siegmund (*1930*), *Sutherland*, *Dunn*, and Maitland (1930), and Lillie (1933)

The Cardiovascular System

Heart The principal changes produced in the heart are dilatation of the right side, hypertrophy of muscle, cloudy swelling of the myocardium, infiltration of the muscle with plasma cells, and subendocardial hemorrhages in the region of the

CHAPTER LXXI

THE VIRUS OF PSITTACOSIS

MORPHOLOGICAL FEATURES

Filtrability and Size

THE VIRUS is not easy to filter through the common types of filter, and there is usually considerable loss of infectivity. It may traverse the following: Seitz EK, Chamberland L_1 bis and L_2, Berkefeld V and N, and Reichel D (Armstrong, McCoy, and Branham, 1930, Bedson, 1930, Elkeles, 1930 *a*, Gordon, 1930 *a*, *b*, Krumwiede, McGrath, and Oldenbusch, 1930, Levinthal, 1930 *b*, Pesch, 1930; Lazarus and Meyer, 1939 *b*).

Direct ... urements have placed the diameter of the coc ... the bacillary forms ranging up to 1000 mμ o ... arus Edlie ...

Electron ...

... 300 mμ ..., and Meyer, 1937, Lazarus and Meyer, 1939 *b*).

Developmental Cycle of the Virus

Bedson and Bland (1932) found that if a mouse was inoculated with a massive dose of virus, the different stages of growth could be observed at will by examination of the spleen at definite intervals In order to demonstrate the virus bodies thin films were made on slides and stained by either the Giemsa or Castañeda method

Fifteen hours after intraperitoneal inoculation of a mouse with a suspension of elementary bodies, the first visible phase of development was the formation of a *plaque* or *plasmodium* inside the cell, which appeared as a light blue or purple-colored homogeneous staining mass.

Twenty-four hours after infection smaller masses developed within the plaques, so that gradually the inclusion body commenced to acquire structural detail, and was now named the *morula* phase of growth As time progressed, distinct elementary bodies began to appear, so that after another 24 hours not only morula phases but also inclusions intermediate between this stage and clumps of elementary bodies were present

At the end of 48 to 72 hours the majority of inclusion bodies had reached the elementary body stage, after which the cell broke down and the virus particles were liberated into the surrounding tissues

Free-lying extracellular virus bodies were usually visible at the end of 24 hours, and these consisted either of fragmented plaques, morulae, or fully developed elementary bodies Elementary bodies were comparatively uniform in size and measured about 0.25 μ in diameter, staining deep pink in color with Giemsa's solution Plaques, however, showed considerable variation in their shape, dimensions, and staining reactions, they measured 1 μ or more in size, showed diplococcal formation, and appeared as diffusely stained structures when seen under the ... scope (see Levinthal, 1930 *b*, 1935 Coles ...

Two theories were originally ... (1933 *a*) in order to interpret the ... by them According to the first o... of the psittacosis elementary bod...

capillary endothelium. Hemorrhages were confined to the Virchow-Robin space, and large phagocyte cells containing fat and hemosiderin appeared in the sites of old hemorrhages. Sprunt and Berry referred to these latter as cerebral purpura, and maintained that the lesions were caused by a toxic change which was secondary to the presence of pneumonia. These same workers also considered that the demyelination reported by Freeman (1933) and Peterson *et al.* (1930) was due to extensive perivascular hemorrhage, and not in the same category as demyelination evident in postvaccinal encephalitis.

Polayes and Lederer (1932) have given a good account of the postmortem findings in the spinal cord in a fatal case of psittacosis which they studied The dura and pia were congested, and on transverse section the posterior cord showed yellowish discoloration. Celloidin and paraffin sections were prepared, and histologically there were p [illegible] anterior horn cells, alteration of the r [illegible] unded and distorted forms possessing [illegible] poorly, and areas of perivascular infiltration and congestion of the blood vessels Hemorrhages occurred into the anterior horn cells and in preparations stained by sudan III small droplets of fat were detected inside the neurons.

LABORATORY DIAGNOSIS OF HUMAN PSITTACOSIS

This is established by inoculating sputum in mice (Rivers and Berry, 1935, Bedson, 1937 *b*), or other animals, or in yolk sacs Lung puncture can be used to obtain material (Yeatman and McEwin, 1945). Tornack (1941 *a*) describes the examination of sputum for EB's using fluorescent microscopy. Advantage may be taken of the bacteriostatic properties of sulfadiazine and streptomycin, which can be added to preparations of sputum prior to inoculation (see Morgan and Wiseman, 1946 *a*).

Failing recovery of virus, acute and convalescent phase sera should be examined by the CFT, more fully discussed below.

This was proved by comparing the relative virulence of 2 types of virus suspension, the one consisting of an emulsion obtained from mouse spleen 48 hours after infection, which consisted mainly of elementary bodies, and the other a similar preparation made 16 hours after infection in which the larger forms of the virus preponderated The results showed that when normal mice were injected with each of these suspensions, death occurred more rapidly in the case of the animals which had received the elementary body suspension than those receiving the emulsion consisting of the large forms. Bedson and Bland point out that "this lower virulence [illegible] normal or [illegible] multipli- [illegible] lty which [illegible] ssential to their growth."

Some Sites in Which Inclusion and Elementary Bodies Occur

Acording to the investigations of Lillie (1930), psittacosis virus is primarily-an-epithelial parasite which is usually found in the microphage and reticulo-endothelial series of cell In man, the inclusion bodies are most evident in the epithelial lining of pulmonary alveoli and also in the periportal connective tissues In the liver they occur inside the Kupffer cells, and in the spleen within the splenic sinuses and macrophage cells of the organ (see Russell, 1931, and Armstrong, 1930) In the case of the parrot, Lillie (1933) reported the presence of elementary bodies within the epithelial cells of the small intestine, ureter, renal secreting tubules, and the bile ducts Inclusions were also found within the epithelioid, macrophage, reticulo-endothelial, and mesothelial cells of these birds In parakeets they were present in the epithelial cells of the renal collecting tubules (see also Rivers, Berry, and Sprunt, 1931). According to Hoge (1934*a*), elementary and inclusion bodies were more numerous in the tissues of the pocket gopher than in any other species of animal susceptible to psittacosis

Inclusions are particularly well seen in the endothelial cells lining the yolk sac It has been found that ether-treatment causes disappearance of the large forms of inclusion, leaving the EB's intact (Kurotchkin *et al*, 1947). Inclusions are also found after amniotic injection, in the tracheal exudate, and superficial epithelial cells of the larger bronchi (Burnet and Foley, 1941) Inclusions have been demonstrated in tissue culture (see above)

TISSUE CULTIVATION

The virus was grown by Bedson and Canti (1935) in their study of the life cycle of the virus (see above). Haagen and Crodel (1937) used the Maitland technique The virus will grow in Li and Rivers' medium, and on Zinsser's solid medium (Yamamura and Meyer, 1941), as well as in roller tube tissue cultures (Morgan and Wiseman, 1946 *b*)

In a series of interesting experiments MacCallum (1936) proved that the presence of living cells was essential for the multiplication of psittacosis virus. Thus, it was shown that the virus failed to grow in cell-free media consisting of chick embryo extract, and even the diffusible growth products of a living tissue culture failed to induce proliferation of the virus in a medium free from cells For the latter experiments, MacCallum employed a principle similar to that adopted in the past by Muckenfuss and Rivers (1930) and Muckenfuss (1931), who devised the method for their investigation into the cultural requirements of vaccinia virus

GROWTH IN THE EGG

The virus grows on the chorio-allantois (Burnet, 1933, Burnet and Rountree, 1935), and multiplies in the ectoderm There is no loss of infectivity for mice on

nass or *plasmodium.* This structure later divided turn subsequently redivided to produce elementary the parasite were structureless in their appearance, Bedson and Bland (1932) considered that at the commencement of its growth the behavior of the virus resembled that of a protozoal parasite rather than that of a bacterium. Subsequent researches by Bedson and Bland (1934) proved this view to be untenable, for it was shown that, although the initial plaques appeared to be homogeneous in structure, they were in reality composed of smaller particles

The true structure of these seemingly homogeneous forms was demonstrated by several different procedures, such as, for example, examining fresh material by darkground illumination, the application of supra-vital staining with brilliant cresyl blue, or the prolonged differentiation of Giemsa-stained preparations with alcohol

In consequence of these later findings, Bedson and Bland (1934) have rescinded their original verdict and offered a second explanation, in which they have maintained that the virus of psittacosis is a micro-organism exhibiting bacterial affinities and not protozoal characters, as was prevously believed

Levinthal (1935) does not agree with Bedson and Bland that the large forms of the virus represent an initial growth phase. He maintains, on the contrary, that when the virus enters a healthy cell the cell resists the invader, and in so doing causes the virus particles to swell up and form a composite deeply staining mass which is devoid of any structure. When the resistance of the cell has been overcome, division and multiplication of the virus then proceed rapidly so that eventually the whole cell becomes packed with elementary bodies Should, on the other hand, the virus enter a damaged cell which is unable to react against it, a prompt and unchecked division of the virus takes place, so that from the beginning of its growth the colony is composed of minute elements No initial large plaque stage of growth would, therefore, be seen if the virus were to enter and develop in a damaged cell

Levinthal also refers to certain forms of the virus, such as rings, disks, and barrel-shaped rods with bipolar staining which resemble thick pasteurella-like organisms, as being degenerate involuted forms of the virus

Bland and Canti (1935) grew the virus in tissue cultures of either fibroblasts or epithelial elements of 9–12 day chick embryos The development of the virus was studied by making stained preparations, as well as by means of the cinephotomicrograph

1 In the early stages, no intracellular inclusions could be seen, but there were numerous EB's

2 From 8–24 hours after infection, round or ovoid intracellular masses were seen, measuring 5–10 μ These bodies were shown to be composed of a mass of bodies 1 μ or less in diameter

3 18–24 hours after infection large masses appeared within the plaques

4 After from 24–48 hours particles varying down to EB's in size developed After 48 hours numerous EB's were seen

5 Between 48 and 72 hours, EB's became the predominant structures They drew attention to a membrane, most readily seen by darkground illumination, encapsulating the colonies In the early stages, the capsule is thick, but gets thinner later This capsule was also noted by Meyer, Eddie, and Foster (1935) in infected sparrows, and somewhat resembles that found in molluscum bodies (van Rooyen, 1938)

Similar developmental forms occur in the infected yolk sac

Observations on the Comparative Pathogenicity of Large and Small Growth Phases of the Virus

Bedson (1933 a) and Bedson and Bland (1934) have drawn attention to the fact that the large forms of the virus are less infective to mice than the smaller varieties

fact that whereas lung changes are the predominant feature of human psittacosis they are relatively uncommon in the parrot (see Heymann, 1930).

Microscopically, the Kupffer cells of the *liver* are filled with hemosiderin and their cytoplasm may contain large basophilic inclusion bodies or numerous elementary bodies, also the bile ducts are damaged and the liver cells necrosed. The *spleen* is infiltrated with lymphocytes and its pigment content is greatly increased. The *kidney* shows focal areas of lymphocytic infiltration and desquamation of tubular epithelium

Lungs. As mentioned earlier, the lungs show comparatively few changes, and this applies not only to parrots which have been spontaneously affected but also to those which have been experimentally inoculated (see Rivers and Berry, 1931, and Lillie, 1930, 1933). In the case of parrots which have been experimentally infected by intramuscular injection of virus into the pectoral region, degeneration and necrosis at the site of inoculation have been described by Levinthal (1930 *a*)

For further details regarding the morbid anatomical and histological features of psittacosis in birds of the species *Amazona amazonica* and *A farinosa* the reader, is referred to the work of Meyer (1931).

Other Birds

Chickens have been experimentally infected by Bedson (1930) who stated that 5 to 9 days after inoculation the animals showed signs of ill health

The *Japanese rice bird* was proved to be susceptible by Levinthal (1930 *b*) who employed it for diagnostic purposes Meyer and Eddie (1933 *b*) likewise obtained positive results with the same bird The *canary* is also receptive to the virus (see Elkeles, 1930 *b*, and Levinthal, 1930 *b*).

The *budgerigar* was found to develop the disease by Bedson (1930), who reported that this species was more resistant than the parrot. Tornack (1941 *b*) discusses the laboratory diagnosis of infection in these birds

Mice

The extreme susceptibility of this experimental animal to parrot strains of psittacosis virus was originally stressed by Gordon (1930 *a*), who in his publication stated that "the outstanding feature of the experiments reported in this paper is the manner in which the strains of virus obtained from parrots associated with the second case of psittacosis have maintained and even increased their virulence by passage through mice" On the other hand, strains of virus isolated from human beings did not appear to be highly pathogenic for mice, and referring to this point Gordon remarks that "the virus derived from human cases has not so far displayed the same degree of pathogenicity, capacity, or stability for mice as that derived from parrots" A similar observation regarding the latter feature was made by Krumwiede *et al* (1930) and Bedson (1930), who found that inoculation of human material into mice did not always produce a fatal illness Bedson showed, however, that the virulence of human strains of psittacosis virus could be exalted in pathogenicity towards mice by first passing the material through birds such as hens, parakeets, or parrots Rivers and Berry (1931 *a*) confirmed these findings.

Following intraperitoneal injection, mice usually die in 6–10 days, but death may be delayed for 3–4 weeks (Bedson, 1930) Mice may appear well, yet be latently infected (Meyer and Eddie, 1933 *b*) Peritonitis is found at autopsy, the spleen is enlarged and shows necrosis and cellular infiltration Inclusions are found in macrophages in the spleen, in the peritoneal exudate, pericardium and pericardial exudate (Levinthal, 1935)

Mice can also be injected intravenously. The cerebral route is effective (Gordon, 1930 *a*, Rivers and Berry, 1931 *a*), and the animals die of meningo-encephalitis in 3–10 days

prolonged passage. Lazarus and Meyer (1939 *a*) reported death of the embryo between the 3rd and 4th day.

It also grows in the amniotic cavity (Burnet and Foley, 1941), and allantoic cavity (Williams, 1944, Francis and Gordon, 1945; Labzoffsky, 1946). The yolk sac is, however, the optimum site for inoculation in the egg when abundant virus is required.

RESISTANCE OF VIRUS TO PHYSICAL AND CHEMICAL AGENTS

Heat. Exposure of the virus for 30 minutes at 55° C. partly destroys but does not completely inactivate it (Gordon, 1930 *a*). Heating to higher temperatures rapidly kills, e.g, 30 minutes at 80° C (see Gordon, 1930 *a*, and Bedson, 1930).

Cold. The pathogenicity of infective material is not altered by freezing the tissues until they turn solid. Bedson (1930) reported that the virus could be preserved in this manner for a period of 10 days.

Desiccation. The virus resists drying and may be preserved for a number of days by subjecting infected mouse spleen to desiccation *in vacuo* over sulfuric acid (Gordon, 1930 *a*).

Centrifugalization at 5,000 r.p m. for 2 hours has been shown to deposit the virus (Bedson and Western, 1930), a further indication of its large size (see also Levinthal, 1935).

Glycerol Bedson (1930) has found that the pathogenicity of infective tissues can be conserved by placing the material in a solution of 50 per cent. glycerol made up in M/50 phosphate solution of pH 7.6. The material was placed in the refrigerator at 34° F., and was found to retain its potency for 20 to 40 days.

Phenol. The virus resists 0 5 per cent. phenol for 20 hours at 37° C, but in connection with tests with phenol, Gordon (1930 *a*) has emphasized the necessity for caution in the interpretation of results owing to the toxicity of 0 5 per cent. phenol solution *per se* to mice.

Ether. The virus was not affected by the addition of 5 per cent. ether to tissues containing it, but the use of 10 per cent. ether caused considerable attenuation in virulence (Gordon, 1930 *a*).

Potassium permanganate. Gordon (1930 *a*) found that a 1 in 10,000 dilution of this substance had no effect on the virus when allowed to act for 30 minutes at room temperature

Other agents The virus is insusceptible to the virus inactivating agent, saponin, sodium desoxycholate, or sodium lauryl sulfate (Burnet, Lush, and Jackson, 1939, Burnet and Lush, 1940) Aerosolized virus is reduced in infectivity by exposure to a cloud of triethylene glycol (Rosebury *et al*, 1947 *b*).

PATHOGENICITY TO ANIMALS

Parrots

Parrots which have not previously suffered from an attack of psittacosis are susceptible to experimental inoculation with virus, when administered either orally, parenterally, or via the respiratory tract

The incubation period varies considerably and ranges from a few days to several weeks, and the principal signs of illness are loss of appetite, diarrhea, disinclination to move, watering of the eyes and nose, with fits of shivering, dyspnea, and convulsions The coat becomes ruffled, the feathers molt, and the bird rapidly loses weight and dies (see Embden and Adamy, 1930) At autopsy, *naked-eye* there are present congestion of abdominal organs, hemorrhages into the myocardium and peritoneum, enlargement with softening of the spleen, miliary nodules in the liver, and fluid in the duodenum with congestion of the pleural cavities Swelling and distension of the upper gut has been described by Bedson (1930) In long-standing cases small foci of consolidation may appear in the lungs, but it is a noteworthy

sided without vesiculation or traces of scar formation (see also Rivers, Berry, and Rhoads, 1930).

Other Rodents

Attempts to infect the wild rat (*Rattus norvegicus*) have been unsuccessful. Likewise similar results were obtained with the Southern California ground squirrel, *Citellus beecheyi beecheyi* Richardson (see Hoge, 1934 *a*).

Some strains are infective for white rats, and the nasal route is more effective than the peritoneal (Chialvo and Parodi, 1944). Cotton rats and hamsters can be infected.

Monkeys

Rivers and Berry (1931 *c*) reproduced lung lesions resembling those found in human psittacosis, by inoculating *Macacus rhesus* monkeys either intratracheally or intranasally with the virus If inoculated by the intracerebral route then meningo-encephalitis accompanied by a mononuclear cell infiltration of the meninges resulted

IMMUNITY

Antigen-Antibody Reactions

Complement fixation has been most studied, and has been widely used in the diagnosis of human and avian infections Bedson (1936) showed that complement fixation is due partly to EB's and partly
terial, there are 2 antigens, one resistant
corresponding antibodies Bedson (1937 *a*
tissue as antigen (see also Meyer and Eddie, 1939 *a*) More recently other antigens have been used tissue cultures (Meyer, Eddie, and Yanamura, 1939, Meyer and Eddie, 1939 *a*), allantoic fluid (Williams, 1944); antigen prepared from the yolk sac by formalin, ether, and centrifugation (Smadel, Wertman, and Reagan, 1943)

Virus neutralizing antibodies develop irregularly, and test mixtures should be injected in mice peritoneally rather than nasally (Rudd and Burnet, 1941).

Opsonization of virus has been shown (Meyer, 1941 *a*).

Agglutination of EB's can be shown, and heat-stable and heat-labile antigens can be demonstrated (Bedson, 1932, 1933 *b*, Lazarus and Meyer, 1939 *c*, Labzoffsky, 1946)

A heat-stable *precipitin* and *allergen* is present in the supernatant fluid remaining after heating a suspension of virus (Bedson, 1936)

Active Immunity on Recovery from Infection

Animals that recover from infection are resistant to a second inoculation In many such cases, the immunity is associated with the continued presence of the virus in the body of the animal (see Meyer and Eddie, 1933 *b*) Certain animals, e g, guinea-pigs, can be hyperimmunized by repeated injections of live virus by a route that does not cause a fatal infection (Bedson, 1933 *b*)

In *man*, second attacks are unusual, but have been reported (Wenckebach, 1936) It is probable that persons frequently exposed to infection become subclinically infected, carry the virus, and become resistant to further attack (see, e g, Gerlach, 1936 *b*)

Active Immunity by Vaccination

Animals, especially mice, can be partially immunized by repeated injections of killed virus The following preparations have been used formolized virus (Bedson, 1933 *b*, 1938), virus inactivated by methylene blue (Levinthal, 1935), formolized tissue cultures (Morgan and Wiseman, 1946 *b*), etherized yolk preparations, egg fluids, and embryo tissue (Wagner *et al*, 1946) It has been found that the peritoneal route is more effective than the subcutaneous Resistance to peritoneal chal-

On nasal inoculation, they develop pneumonia, with EB's in the alveolar and bronchial lining cells, titrations can be performed by counting the focal lesions (Hornus, 1940); Rudd and Burnet, 1941).

Pocket Gopher

The pocket gopher or *Thomomys bottae bottae* is reported by Hoge (1934 *a*) to be more susceptible than the white mouse.

The animals can be infected by any route of experimental inoculation and the pathological lesions produced are constant in character. A subcutaneous dose of 0 2 c c. of a 1 per cent. suspension of virulent mouse spleen which is uniformly lethal to mice in 7 to 8 days usually kills gophers after 3 to 5 days' time. The virus may be serially passaged through these animals, and their tissues are infective to mice. Repeated passage in gophers is said to reduce temporarily the killing time for mice, but this may be restored by successive passage through mice.

At autopsy there is extensive hemorrhagic infiltration of the abdominal wall and peritoneum (following inoculation by any route of administration), enlargement of the liver, and softening of the spleen Microscopically, areas of coagulative necrosis occur in the liver, the cells are oxyphilic in character, their nuclei show karyolysis and karyorrhexis, and there is swelling of the Kupffer cells. The spleen shows areas of caseation and thrombosis, the lungs are hemorrhagic and edematous, and the heart muscle and the kidneys show evidence of cloudy swelling. Impression preparations made from the liver and spleen, when stained by Romanowsky methods, reveal the presence of innumerable LCL bodies, greatly exceeding in number those found in similar preparations made from mice Lillie and Hoge (1934) remark that they can find no evidence to suggest that gophers suffer from psittacosis under natural conditions

Rabbits

Rabbits are susceptible to infection by intracerebral inoculation, and the lesions produced in the central nervous system of these animals have been described by Gordon (1930 *a*) A strain of psittacosis virus which had been isolated from a sick parrot and passaged in mice was inoculated into the brain of a rabbit, and 4 to 7 days later the animal developed signs of meningitis and died At autopsy no organisms could be cultivated from the meninges of the animal. Microscopically, there were seen lymphocytic infiltration and thickening of the meninges, with areas of perivascular "cuffing" surrounding the vessels of the white matter of the brain. Small foci of inflammatory reaction were scattered throughout the organ and there was pronounced degeneration of nerve cells A certain proportion of rabbits inoculated intracerebrally tend to recover from the infection, and unless the virus is passaged within the first week after inoculation it may be lost Rivers and Berry (1931 *b*) found that 4 to 5 days after injection the temperature of the rabbit rose to 104° F. or more, after which it returned to normal by the end of the second week, thereafter the animal recovered completely and became immune to re-infection.

Guinea-pigs

Guinea-pigs were also injected intracerebrally and shown to develop signs of meningo-encephalitis Rivers and Berry (1931 *b*) found that passage of psittacosis virus through guinea-pigs or rabbits did not diminish its pathogenicity for parrots or mice Bedson (1930) found that if guinea-pigs were inoculated intraperitoneally with virulent material they harbored the virus for a short time, and it could be recovered from their tissues by inoculation of mice Injection of psittacosis virus into the pad of the guinea-pig's foot resulted in the formation of redness and swelling which attained a maximum about the 3rd or 4th day, and thereafter sub-

Faroe
moner
petrels
–Sept.
16th, and the human cases occurred between the 30th and 25th of these months (Rasmussen, 1938 *a*, *b*, see *Brit. med. J.*, 1939, *1*, 735) Virus was isolated from the birds by Haagen and Mauer (1938). Since then, numerous cases have been reported, chiefly following exposure to pigeons, less commonly to doves, or chickens (Meyer, 1941 *b*, Alicandri, 1942, Meyer and Eddie, 1942, Meyer, Eddie, and Yanamura, 1942 *a*, Levinson, Gibbs, and Beardwood, 1944, Melamed and Fine, 1944; Meiklejohn, Beck, and Eaton, 1944, Eaton, 1945, Cohen, Gray, and London, 1946).

Infection in pigeons is widespread and has been demonstrated in birds caught in America, Canada, England, S. Africa, and elsewhere (see e g, Coles, 1940, Andrewes and Mills, 1943, Levinson, Gibbs, and Beardwood, 1944, Labzoffsky, 1947). The diagnosis is made by CF tests or isolation of virus

Turkeys, ducks, and chickens have also been found infected, but not wild fowl (Eddie and Francis, 1942).

Infection in pigeons may be acquired in the nest or shortly afterwards. The birds develop a latent infection, and become sick if subjected to overcrowding and other adverse conditions (Meyer, Eddie, and Yanamura, 1942 *a*). The virus, although in general very similar to psittacosis, can be distinguished from it. It infects mice only cerebrally or nasally, and not regularly peritoneally or subcutaneously (Pinkerton and Swank, 1940, Meyer, Eddie, and Yanamura, 1942 *a*). It also infects ferrets, cotton rats, and hamsters nasally, guinea-pigs and monkeys develop focal

toic cavity

The virus is sensitive to penicillin *in vivo* (Heilman and Herrell, 1944 *a*, Turgasen, 1944) The relationship of the virus to other members of the pneumonitis group is discussed in Ch. LXVII.

THE SÃO PAULO PARROT DISEASE

The São Paulo parrot disease was discovered by Pacheco and Bier (1930) and Meyer (1930) during the course of their investigations into an outbreak of psittacosis which affected an aviary at Agua Branca in the State of São Paulo in 1930. The viruses responsible for these two conditions have been compared as follows by Pacheco (1932)

The Virus of Psittacosis	The Virus of São Paulo
Is infective to man, monkey, mouse, rabbit, hen, and canary	Is only pathogenic to psittacidae of the genus *Amazona*
Exhibits neurotropic affinities	Is viscerotropic in character
Forms basophilic intracytoplasmic inclusion bodies	Forms acidophilic intranuclear inclusions (see Rivers and Schwentker, 1932)
Is not destroyed by dessication	Is killed by drying
Duration of illness is 4 to 5 days	Duration of illness is 2 to 8 days
The principal naked-eye pathological lesions are congestion of the intestines and splenomegaly	Areas of focal necrosis are found in the liver, spleen, and lung, congestion of the intestines is sometimes observed
During an attack of the disease the animal shows signs of inactivity and drowsiness accompanied by diarrhea, oculonasal discharge, coughing, and coma before death	Inactivity, loss of appetite, drooping of the wings, whitish or blood-stained diarrhea, and coma are usually present

Both diseases are caused by filtrable viruses which resist glycerol and are not destroyed by heating at 55° C.

lenge is acquired much more easily than resistance to cerebral or nasal challenge (Rudd and Burnet, 1941; Wagner *et al*, 1946).

Although no clinical evidence of illness may follow the challenge, in many cases a latent infection is set up and virus is demonstrable in the tissues (Bedson, 1938, Yanamura and Meyer, 1942).

Although most experiments have been performed with mice, birds such as parakeets and rice birds can also be immunized (Meyer, Eddie, and Yanamura, 1942 *b*).

In *man*, Rivers and Schwentker (1934) inoculated volunteers intramuscularly with suspensions of live virus. There were no ill effects, and antibodies developed.

Serum Antibodies

Antibodies develop in animals naturally or experimentally infected. In man the complement fixing antibody has been most studied, as virus neutralizing antibody is difficult to demonstrate (Rivers and Berry, 1931 *a*; Bedson, 1933 *b*). The complement fixation test, using antigens already described, may first become positive as early as the 8th to 12th day of illness, but may appear later, a definite rise in titer should, of course, be demonstrated between acute and convalescent phase sera (Pye-Smith, Guest, and Bedson, 1933; Bedson, 1935; Fortner, 1936, Meyer, 1940, Sordelli and Savino, 1940, Favour, 1943).

It must be realized that a positive reaction in the CFT only indicates infection by a member of the LGI-psittacosis-pneumonitis group (see Ch LXVII)

Passive Immunity

Convalescent serum has been recommended for trial in cases of human infection (Meyer and Eddie, 1939 *a*).

ACTION OF CHEMOTHERAPEUTIC AGENTS AND ANTIBIOTICS

Chemotherapeutic and antibiotic agents have been shown to have some effect against psittacosis virus. Thus, *sulfadiazine* given in the diet saves mice from death, but they become carriers (Early and Morgan, 1946 *b*). The drug has a marked inhibitory effect on the growth of some strains of virus in eggs (Early and Morgan, 1946 *a*; Meiklejohn, Wagner, and Beveridge, 1946).

PABA given in the diet did not save mice (Early and Morgan, 1946 *b*), nor was it effective in eggs (Early and Morgan, 1946 *a*)

Large doses of *penicillin* subcutaneously save the majority of mice from death, but they usually develop a symptomless infection, and become resistant to reinoculation (Heilman and Herrell, 1944 *b*, Bedson and May, 1945, Early and Morgan, 1946 *b*). Penicillin inhibits virus growth in tissue culture, but is not so effective in eggs (Early and Morgan, 1946 *a*, Meiklejohn, Wagner, and Beveridge, 1946)

Streptomycin has not been found effective in mice (Early and Morgan, 1946 *b*), tissue cultures or eggs (Early and Morgan, 1946 *a*).

Some human cases of psittacosis appear not to have been benefited by sulfonamides (see e g, Toomey and Lohrey, 1946), but others have improved with penicillin and sulfonamides (Rosebury *et al*, 1947 *a*).

INTRANUCLEAR INCLUSION VIRUS

This virus causes intranuclear inclusions of the herpetic type in pigeons There are focal necrotic lesions in the viscera The virus is smaller than psittacosis, and immunologically distinct Pigeons may be infected simultaneously with psittacosis and INI virus (Smadel, Wall, and Gregg, 1943, Smadel, Jackson, and Herman, 1945).

ORNITHOSIS

This term is applied to a closely related infection contracted from sea birds, pigeons, doves, and similar birds.

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CHAPTER LXXII

LYMPHOGRANULOMA INGUINALE (CLIMATIC BUBO)

LYMPHOGRANULOMA INGUINALE (LGI) is the term most commonly used in Britain to describe a disease previously known variously as climatic bubo, venereal bubo, poradenitis, malady of Durand, Nicolas, and Favre, 4th venereal disease, or 6th venereal disease The condition is also known as lymphogranuloma venereum and lymphopathia venerea It should on no account be confused with either granuloma inguinale or lymphadenoma (Hodgkin's disease).

Lymphogranuloma inguinale is an infective disease, usually spread by venereal contact and manifested by a wide variety of clinical features. It is most frequently seen in warm climates and can be diagnosed by cutaneous and other tests A virus which is transmissible to numerous experimental animals can be isolated from the lesions

Stannus (1933) reviewed over 900 papers, and there has been no lessening of interest since that date A numbe
(Coutts and Bianchi, 1934, Wassé
and von Haam, 1939, Favre and
Grace, 1941 *b*, Hickam, 1945).

CLINICAL FEATURES

For many years a disease of the inguinal lymphatic glands known as climatic bubo was familiar to those practicing in the tropics, and was from time to time recognized elsewhere among persons returned from abroad Infection was known to occur following sexual intercourse, but despite this fact almost all the recognized cases were in males.

For some considerable time a very similar condition was also known to workers in cooler lands such as France, Germany, Italy, and Scandinavia, and was named lymphogranuloma by Durand, Nicolas, and Favre in 1913 (*a*, *b*) because of an histological similarity to lymphadenoma. They did not, however, realize that they were dealing with the disease known in warmer lands as climatic bubo Interest was interrupted by World War I, but revived thereafter when the identity of CB and LGI soon became established. Amongst work which contributed to this view may be mentioned the introduction of Frei's cutireaction in 1925, the transmission of the virus to monkey.
diti *et al* in 1932 (*b*).
infected mice with th
in the tropics He was able to show that infection of mice could be prevented by the serum of cases of LGI obtained from Paris

Earlier Manifestations of LGI

The primary sore Following an incubation period of from 3 days to about 3 weeks, the primary lesion develops, usually in the genital region In males it is commonly on the penis, especially the glans In women while it may be on the labia, it is commoner on the vaginal wall or cervix Primaries may occur in the region of the anus The primary lesion may take one of four forms (Chapman and Hayden, 1937) (1) herpetiform and transient, (2) an ulcer, (3) a nodule, (4) a non-gonococcal urethritis (*see below*). Extragenital primaries may occur on the finger, tongue, or tonsil (Klotz, 1890, Ravaut and Scheikevitch, 1921, Buschke and Curth, 1931; Bloom, 1933, David and Loring, 1936, Tanahashi, 1936).

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the aperture. This type of stricture is usually single, but may be multiple, and is not progressive.

2. A funnel-shaped stricture, from 2 to 6 cm. long, with its smallest aperture proximally, may be found, the overlying mucosa is glistening and hyperplastic, while distally it is ulcerated and polypi may be present.

3. This type is found rarely; it occurs where the whole bowel distal to the pelvirectal junction is involved

Clinical features of stricture. In the early stages there is usually a complaint of pruritus and burning in the anal region, the mucosa being swollen and hyperemic (Mathewson). Later, there is bloodstained mucoid discharge, with tenesmus. Constipation and sometimes even acute obstruction may occur. Examination shows the

Michelson *et al.* (1938) recorded a normal delivery in a case of rectal stricture, while Mathewson (1938) has reported that cesarean section may be needed in certain cases Coutts and Monetta (1938) recorded a birth in a case of ulcero-elephantiasis which was normal except for a temporary retention of the placenta.

(*e*) *Involvement of alimentary tract.* Some cases of ulcerative colitis, and perhaps even ileitis, may be due to LGI (Paulson, 1937, 1938, 1939; Whittaker, 1937, Coutts, Opazo, and Montenegro, 1940). Rodaniche, Kirsner, and Palmer (1940), however, did not find evidence of a close association with colitis Coutts

"leather-bottle" stomach.

LGI may cause urethritis, stricture

Polak, 1933, Mauro, 1939, Itikawa and Shinoda, 1939–40, Coutts, 1943 *a*, *b*, Tauber, 1945, Fowler and Walker, 1947). The condition known as Waelsch's "sago-grain" urethritis may in some cases be due to LGI (see Harrison and Worms, 1939, Melczer and Wlassics-Venkei, 1930).

Leukoplakia of the bladder may occur (Carloni, 1944), and chronic cystitis (Coutts and Vargas-Zalazar, 1945).

Epididymitis has been reported (Coutts and Herrera, 1938, Sicard, Leger, and Levaditi, 1945)

Other Manifestations of LGI

It has come to be recognized that LGI is more than a disease localized merely to the pelvic organs, for a variety of other changes may occur (see Coutts, 1936; von Haam and D'Aunoy, 1936 *b*, Chapman and Hayden, 1937, Eberhard, 1938, Jones and Rome, 1938, Gutman, 1939).

1 The throat may be involved, with inflammation of the tonsils, ulceration, or angina Alverson (1941) described a granulomatous condition of the larynx and hypopharynx

2 The patient is often febrile and suffers from headaches and other pyrexial disturbances.

3 Skin rashes have been described (see e g, Hellerstrom, 1929, Lohe and Blummers, 1931, Kleeburg, 1931, Melczer and Sipos, 1938 *b*, Benedek and Olkon, 1941). There seems to be a relationship between LGI and erythema nodosum in some cases (Sonck, 1940 *b*, 1941, Hellerstrom, 1941, Hickam, 1945). Sonck (1940 *a*) refers to the frequency of solar eruptions

4 Various forms of articular affection have been attributed to LGI (Gottlieb, 1932 *b*, Jones and Rome, 1938, Sonck, 1941, Hickam, 1945), e g., arthralgia, polyarthritis, and effusion Osseous changes may occur (Wright and Logan, 1939).

5 There is often generalized enlargement of the lymphatic glands, the liver, and spleen

Lymph gland involvement. The primary lasts for a few days and then the virus spreads by lymphatic vessels to the regional glands, where it causes inflammation and suppuration. The distribution of the pelvic lymphatics in the male and female is of considerable importance in determining the future course of the infection (Barthels and Biberstein, 1931; Nesselrod, 1936 *a*, *b*). In males, the primary lesion being usually on the penis, the inguinal glands are first involved. Suppuration occurs with the formation of large buboes, which may break down to form sinuses. Sometimes, however, the primary may be intra-urethral and infection can then spread from the posterior urethra to the intrapelvic glands (see e g, Mathewson, 1938) If the primary is in the anorectal region, the infection also reaches the pelvic glands (Feilchenfeld, 1932).

Rarely, inguinal buboes may be accompanied by enlargement of glands elsewhere (Costello and Cohen, 1940).

In females, primaries on the external genitalia may give rise to lymph gland involvement in the groin. More usually, however, the primary is intravaginal and infection spreads, posteriorly to intrapelvic glands.

Later Pelvic Manifestations of LGI

The genito-anorectal syndrome. This is the term usually applied to the tertiary stages of LGI and includes a number of disorders attributed to involvement of the lymphatic system and adjacent structures by a chronic inflammatory process. It has been suggested that the syndrome predisposes to malignant change (Coutts, Brieva, and Lerner, 1942).

(*a*) "*Esthiomène.*" The term "esthiomène" is applied to a chronic granulomatous condition of the vulva, which becomes swollen and infiltrated, and fistulae, ulcers, and sinuses may form. The disease is usually associated with other manifestations of infection

(*b*) *Elephantiasis* In certain cases elephantiasis of the vulva without other destructive lesions may occur In the male, elephantiasis of the penis and scrotum has been attributed to LGI (see e g., Stannus, 1933, Stryker and Ploch, 1935). Plastic induration of the penis may be caused by LGI (May, 1944)

(*c*) *Pelvic inflammation* Cases of salpingitis and tubo-ovarian abscess have been attributed to LGI (D'Aunoy and Schenken, 1938, and Franci, 1934, respectively) Kondo (1935) reported that glands might rupture and set up peritonitis

(*d*) *Rectal stricture* The condition of benign rectal stricture has been known to surgeons for many years Formerly attributed to syphilis, traumata, or other causes, it is now recognized that the virus of LGI is the cause of the great majority.

This form of rectal stricture is being increasingly recognized in many parts of the world, and in particular in N America (Frei and Koppel, 1928, Lutz, 1932, Cole, 1933, Bacon, 1935, Lichtenstein, 1936, Chapman and Hayden, 1937, Mathewson, 1938, Peyton, 1940, Grace, 1943, Armstrong and Niebauer, 1944) A few cases contracted in Britain have been recorded (Findlay, 1935–6, Manson-Bahr, 1935–6, Whittaker, 1937).

The lesion is generally thought to be predominantly one of women, especially of the colored races. Certain recent papers, however, have reported the occurrence of strictures in men, and the practice of sodomy accounts for some of these, primary in the anal region giving rise to an intrapelvic spread of virus In other cases an intra-urethral primary has probably been responsible Chapman and Hayden recorded 11 cases in men, and Mathewson no less than 60

Virus may reach the rectum by invasion of the perirectal tissue, or, as suggested by Grace (1941 *b*), by direct implantation of virus on the mucous lining.

Types of stricture. According to Mathewson, who has published one of the most authoritative articles on the subject (74 cases), there are 3 types of stricture·

1. An annular diaphragm of fibrous tissue forms about 1 to 6 cm. above the ano-cutaneous margin. He states that the proximal mucosa may permeate through

the aperture. This type of stricture is usually single, but may be multiple, and is not progressive.

2. A funnel-shaped stricture, from 2 to 6 cm long, with its smallest aperture proximally, may be found, the overlying mucosa is glistening and hyperplastic, while distally it is ulcerated and polypi may be present.

3. This type is found rarely; it occurs where the whole bowel distal to the pelvirectal junction is involved

Clinical features of stricture. In the early stages there is usually a complaint of pruritus and burning in the anal region, the mucosa being swollen and hyperemic (Mathewson). Later, there is bloodstained mucoid discharge, with tenesmus. Constipation and sometimes even acute obstruction may occur. Examination shows the distal mucosa to be papillomatous In the late stages fistulae form and may open into the vagina Often "esthiomène" or elephantiasis is present as well.

Pregnancy and genito-anorectal syndrome. Little has been written about the effect of "esthiomène," rectal stricture, or other manifestation upon pregnancy. Michelson *et al* (1938) recorded a normal delivery in a case of rectal stricture, while Mathewson (1938) has reported that cesarean section may be needed in certain cases. Coutts and Monetta (1938) recorded a birth in a case of ulcero-elephantiasis which was normal except for a temporary retention of the placenta.

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(*f*) *Involvement of urethra and bladder.* LGI may cause urethritis, stricture may occur (Anwyl-Davies and King, 1933, Polak, 1933; Mauro, 1939; Itikawa and Shinoda, 1939–40, Coutts, 1943 *a*, *b*, Tauber, 1945, Fowler and Walker, 1947). The condition known as Waelsch's "sago-grain" urethritis may in some cases be due to LGI (see Harrison and Worms, 1939, Melczer and Wlassics-Venkei, 1939).

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5 There is often generalized enlargement of the lymphatic glands, the liver, and spleen

6. It is probable that vascular disease may be caused by LGI (Coutts and Davila, 1945; May, 1945; Soriano and Yrastorza, 1945).

7. A few cases of meningo-encephalitis have been reported (Rajam, 1936, Sabin and Aring, 1942, Zarafonetis, 1944, Scott, 1945).

8. Various ocular conditions have been attributed to LGI, e g, iritis, keratitis, keratoconjunctivitis, uveitis, Parinaud's syndrome, chalazion, and pterygium (Levaditi *et al*, 1936 *b*, Ouwejan, 1939, Vasquez-Barrière and May, 1940, Benedek and Olkon, 1941; Macnie, 1941, Espíldora and Coutts, 1942; Scheie, Crandall, and Henle, 1947).

9. Pleurisy and ascites may occur (Litarczek and Chisăr, 1939).

10. LGI may be an etiological factor in certain cases of otosclerosis (Chiarino, 1942; Mourao, 1945).

Infection in Children

A few cases of presumably acquired LGI have been recorded, predominantly in the female child, while inguinal adenopathy is common, rectal changes may also occur (Levy, 1937, Sonck, 1939, 1940 *a*, Levy, 1940, Winge, 1941) Dick (1936) described a case of rectal stenosis that may have been hereditary. The Frei test however is usually negative in the children of Frei-positive mothers (see below)

DISTRIBUTION OF THE VIRUS

The primary lesion. Virus has been found in primary lesions (Lohe *et al*, 1933; Shimoda, 1939)

Pus. The virus is regularly found in the pus from inguinal buboes, and it was with this material that the original transmission experiments of Hellerstrom and Wassén, Levaditi, and Findlay were performed

Tertiary lesions A number of workers has been successful in isolating the virus from the rectal tis[illegible]t *et al*, 1932, Laederich *et* [illegible] Jollaret, and Remié, 1935, [illegible] Caminopetros, 1938 *a*) Lo[illegible] a case of "esthiomène"

It should be noted that Caminopetros (1935) isolated virus from the urethral and cervical tissue of prostitutes known to be spreading the disease, although they did not show any manifestations of infection.

Infection of the epididymis and seminal vesicle, and of the ovaries, has been recorded (see Coutts and Monetta, 1938). Virus has been isolated from ascitic fluid in a case of acute peritonitis developing in the course of LGI (Aoyama, 1938)

Blood Virus was isolated in one case (Beeson, Wall, and Heyman, 1946), Findlay (1933–4) obtained negative results

Cerebrospinal fluid Virus has been found in the cerebrospinal fluid (Coutts *et al*, 1933; von Haam and D'Aunoy, 1936 *a*, Jones and Rome, 1938, Beeson, Wall, and Heyman, 1946)

Feces Paulson (1937, 1938, 1939) has reported that the feces of cases of ulcerative colitis, in patients giving a positive Frei intradermal test, may contain the Frei reacting substance, and thus postulates the presence of the virus in the feces of such cases. He has prepared Frei antigen from the feces and produced a positive reaction on intradermal injection of known cases of LGI (see also Paulson and Kravetz, 1938)

CLINICAL PATHOLOGY

Albuminuria is not uncommon There is a slight "secondary" anemia, leukopenia is found in the early stages, but a polymorph leukocytosis occurs at the suppurative stage (Ishizuka, 1938 *a*, Jones and Rome, 1938). The BSR is increased (Nicolau, 1931, Michelson *et al*, 1938, Ishizuka, 1938 *a*, Schmidt, 1938, Gsell, 1939)

Hyperproteinemia, due to increase of globulin, occurs in LGI and parallels the ata tests are usually positive (Gutman Gutman, 1936, Gutman *et al.*, 1936, 9, Longhin and Stoian-Ionesco, 1937, Michelson *et al*, 1938, Gsell, 1939, and Miller, 1944 *a*, Combes, Cañizares, and Landy, 1945). Although the serum protein rises to 10 per cent or more, there is no associated hypercalcemia (Williams and Gutman, 1936).

The lipoid content of the serum is said to be below normal (Rosen *et al*, 1937, 1939), but Jones and Rome (1937) reported a hypercholesterolemia

PATHOGENESIS

loma inguinale is primarily a disease of the

in the female the pelvic, glands are those most commonly involved.

It appears that tertiary lesions are more prone to develop in those who have not shown buboes

The differing clinical picture in the lymph glands and rectal tissue is explicable by the reactivity of these organs, although adenitis heals in a few weeks, the rectal lesion persists (Caminopetros, 1939)

PATHOLOGY

According to Sheldon and Heyman (1947)

ble for the vascular occlusion

A. Primary Lesion

There is little that is characteristic about the primary ulcer. The surface epithelium is denuded, and the ulcer cavity is surrounded by tissue showing a marked infiltration with small round cells, plasma cells, and perhaps histiocytes (see Bory, 1921, Shinoda, 1939)

B. Lymphatic Glands

The histology of the glands has frequently been described (e g, Phylactos, 1922, Hellerstrom, 1929, Thomson, 1936, Hiraga, 1937, Anderson and Harmos, 1938, Tanahashi, 1938, Sepulveda, 1942)

The glands are matted together by much fibrous and granulation tissue. In the more advanced cases suppuration is present and the glands may be adherent to the overlying skin, with fistula formation On section, the periglandular tissues show an inflammatory reaction more or less acute, and the gland capsule is thickened. The gland substance shows numerous small lesions scattered throughout. In the early stages there is much cellular proliferation with formation of small nests of mononuclear cells. Later these nests become necrotic in the center, polymorphs appear and small abscesses, which eventually coalesce, result. These abscesses are surrounded by

be found

The follicu

material ca

observed In certain cases the glands become fibrotic, and in the dense material occur small abscesses In a case examined post mortem by Reichle and Connor (1935) the entire retroperitoneal chain of glands was involved in the process.

C. Tertiary Lesions

In males, the primary lesion is so situated that the glands affected are the inguinal. In the female, however, owing to the usual site of the primary, the affected glands are intrapelvic. In the male, suppuration occurs and the disease tends to limit itself, tertiary manifestations being rare. In the female, however, the infection spreads in the pelvis and pudenda and may cause two main types of tertiary lesion, often classed together as the genito-anorectal syndrome.

"Esthiomène." This term includes a variety of lesions of the vulva The labia are usually affected, being greatly thickened, nodular, and edematous, ulceration is frequently superimposed and a urethral or rectal fistula may form Condylomata may be found around the urethral or anal orifices Histologically, the changes are of the same nature as those found in the glands. The thickening of the parts is due to newly formed granulation tissue in the dermis, and there is a marked cellular infiltration with lymphocytes, histiocytes, polymorphs, and a number of giant cells. Small abscesses may be found resembling those described in the glands.

Rectal stricture. This is predominantly a disease of women, the ratio being probably 4 or 5 cases in women to every 1 in men. As has already been noted, however, cases in men are not uncommon. The stricture is usually situated a few centimeters above the anus. It is most frequently of the ring type and is often associated with [illegible]
ulceration of th [illegible]
replacement of [illegible]
with lymphocytes, plasma cells, and endothelioid cells (e.g, Barthels and Biberstein, 1931; Lichtenstein, 1936). Small abscesses may also be found. The mucosa is ulcerated, and on regeneration there is a tendency to squamous metaplasia. Falconer (1938) found very similar changes in a case of colitis in a patient who had LGI 15 years before. Lichtenstein reported that the final stage of LGI infection is the condition known to surgical pathologists as "chronic ulcerative stenosing proctitis and periproctitis"

D. Other Changes

Reichle and Connor (1935) found a psoas abscess, venous thrombosis in the adrenals, and a proliferative glomerulitis. Wohlwill (1943) concluded that the virus causes a general activation of the reticulo-endothelial system, in one case he found granulomatous lesions in the spleen

E. Inclusion Bodies

Gamna (1923, 1924) and Favre (1924) described inclusions (Gamna-Favre [illegible] ionuclears, and polynuclears, in lymph [illegible] nd are spherical, cone, or dumb-bell [illegible] be surrounded by a vacuole. Findlay (1933–4) found that they gave a positive Feulgen test, a negative test for fat, and did not stain with Janus green B for mitochondria, thus proving their nuclear origin. The present opinion is that Gamna-Favre bodies are of nuclear or nucleolar origin, some being the remains of phagocytosed lymphocyte nuclei, and others nucleolar extrusions. Whatever their origin, they are certainly of diagnostic value (Todd, 1926).

Miyagawa *et al.* (1935 *a*) have described quite different inclusions which they [illegible] These measure [illegible] They may be [illegible] ves no reaction (see also Robinow and Bland, 1938), the bodies are not stained by Gram's method These granules are found in varying numbers in the cytoplasm of histiocytes, and sometimes lymphocytes and polymorphs of human lymph glands Cells around the

small localized abscesses contain more than do other cells Cells containing these structures show no signs of degeneration unless they are present in very large numbers Similar bodies are also found lying free, often seeming just to have ruptured from a cell. These granules have also been found by Satani and Sano (1936), Mauro (1937); Melczer (1938); Caminopetros (1938 *a*), Coutts and Herrera (1938), Pirilla (1942).

Coutts and his collaborators (Coutts, 1946) have found inclusions in small groups or clusters varying from 0 2–2 μ in size in material from the buccal cavity, vulva,





Fig 38 Fig. 39

Figs. 38 and 39 Inclusions of lymphogranuloma inguinale. Fig 38 shows morula type of inclusion in rectal tissue, Fig 39 shows inclusions in tissue in a case of ptyergium

(By courtesy of Dr W E. Coutts, Santiago, Chile)

vagina, urethra, and rectum Small forms 0.2–0 8 μ are usually found free and in large numbers Larger forms up to 1 μ appear free or in the cytoplasm of histiocytes, singly, in pairs, or clusters Forms resembling Russell bodies, 1–3 μ in diameter are found free or in histiocytes These bodies resemble those described by Ishimitsu (1936, 1937). Although a number of authors has disputed the specificity of the Russell body type of inclusion, Coutts is convinced of their viral nature, and finds them more frequently in tertiary lesions Coutts and his collaborators have found inclusions in lung tissue, blood vessels, stomach, bladder, conjunctiva, lacrimal duct and sac, and gallbladder, in addition to the better-known lesions (see Figs 38 and 39)

EPIDEMIOLOGY

Method and Source of Infection

By far the commonest method of infection is by sexual intercourse. The disease is especially prevalent among colored prostitutes Although many of these suffer from the genito-anorectal syndrome, it appears that in certain cases the disease may be transmitted by one who seems to be free from clinical signs. Further, Caminopetros (1935) isolated the virus from the urethra and cervical canal of such persons, although they showed no evidence of abnormality. Studies of the Frei reaction have shown that in certain areas up to 40 per cent or more of these women may react positively. Thus in St Louis it was found that 47 7 per cent of colored prostitutes reacted positively to this test (Gray *et al*, 1936) In Brazzaville (French Congo) 43 5 per cent gave a positive Frei test (Saleun, Ceccaldi, and Gourvil, 1936) Sorel (1936) in French Equatorial Africa found that 27 out of 68 such persons showed a positive test (for similar reports see Vaucel *et al*, 1936,

Histologically, the brains of infected monkeys show a diffuse leptomeningitis In the acuter cases the leptomeninges are infiltrated with a fibrinous exudate in which are found polymorphs. Perivascular cuffing is a prominent feature. In chronic cases the cellular infiltration is composed more of lymphocytes, plasma cells, and macrophage cells which may show Gamna-Favre bodies or granulocorpuscles.

Hellerström and Wassén were also able to infect monkeys by intrapreputial and intraperitoneal injections. It has been reported that after injection of virus intracerebrally or intraperitoneally pseudotabetic manifestations may develop, with histological changes in Goll's column (Jonesco-Mihaiesti *et al.*, 1932 *a, b*, 1933 *a, b*). On peritoneal injection a cellular exudate may form (Mesrobeanu, 1938, 1939) Findlay (1933–4) carried out injection into the groin. Injection into the wall of the intestine or rectum may secure a positive result (Levaditi and Reinié, 1934, Levaditi, Mollaret, and Reinié, 1935), as may intrapulmonary injection (Caminopetros, 1934). Findlay (see 1935–6) injected the tissues anterior to the rectum. A typical inflammatory reaction developed locally, and the virus was isolated from enlarged inguinal glands.

2. Mice.

The high price of monkeys renders them unsuitable for use in large numbers Fortunately, however, it was discovered by Levaditi *et al* (1932 *b*) and Findlay (1932) that the disease was transmissible to mice by intracerebral injection. These observations have been confirmed by various authors (D'Aunoy and von Haam, 1936 *b*, Mesrobeanu and Bruckner, 1936, Cottini, 1938 *b*, Gavrilof and Fester, 1939) *The infected animals develop a characteristic meningitis in 7 to 14 days as shown* by muscular incoordination, paresis, and weakness. Two strains studied by Findlay (1938) produced symptoms in 2 to 4 days Conjunctivitis may also occur.

The histological appearances are similar to those of the monkey, and granulocorpuscles can be found. Granulocorpuscles are most abundant between the 5th and 20th days, and occur especially in the ependymal cells covering the infundibulum of the 3rd ventricle, and in monocytes in the ventricular exudate (Levaditi, 1944 *a, b, c*), histological changes can be found after 3–4 days (Levaditi, 1944 *c*) Mice sometimes show no clinical evidence of infection following intracranial injection, Levaditi and Schoen (1936) found a hydrocephalus to be present in certain of such animals. Virus may persist for a very prolonged period in the brain in inapparent infections (Levaditi, 1944 *d*, Wall, 1947). Virus can be found in the CSI (Okanishi and Vio, 1937)

Mice may be infected by the intracutaneous and intratesticular routes also. They can be infected intraperitoneally if starch is injected intracerebrally (Findlay, 1933–4)

The virus can be passed by
consolidation occurs in 48–72
in the alveolar lining (Schoen,
and O'Donnell, 1944, Landau
solidated foci visible *in situ;* 1
mum concentration by the 31
mice are infected, *the virus is transmitted to the fetus (Hellendall, 1942, 1945)*

Findlay (1938) has reported that strains of virus may vary considerably in the ease with which they infect mice Thus, of 26 strains transmitted by him to mice only 2 proved highly virulent, while others lost their virulence with passage

3. Guinea-pigs.

Following injections of infective material into the groin, between one-third and one-half of animals develop enlarged inguinal glands This was probably first shown by Gamna (1923, 1924), and it has since been confirmed by other workers (see,

e g, Meyer, Rosenfeld, and Anders, 1931; Freund and Reiss, 1931), most of whom stress the fact that 3 or 4 animals should be injected, in order to secure at least 1 positive result The histological picture shows a marked reticulo-endothelial response. Sections of the infected gland show a great increase in the number of histiocytic cells, many of these containing Gamna-Favre bodies In many cases

[illegible] hocyte nuclei, as consid- [illegible] Guinea-pigs can also be [illegible] gawa *et al.*, 1936 *a*, Grace [illegible] d Sano, 1936, Grace and [illegible] ros, 1934), intratesticular [illegible] perianal region (Caminopetros, 1938 *a*), into the vaginal mucosa, or into the prepuce (Grace and Suskind, 1940).

Some workers have failed to infect using the intracerebral route, but others have produced meningo-encephalitis and found granulocorpuscles (Coutts *et al*, 1939, 1942 *a*).

The nasal and peritoneal routes are not effective (Schoen, 1939 *d*, Grace and Suskind, 1940, Beck, Eaton, and O'Donnell, 1944).

Histological changes have been recorded in the kidneys in infected guinea-pigs (Coutts, Brieva, and Lerner, 1943).

Other animals.

[illegible] the

[illegible] ijections

5. *Cats*, by intracerebral injection (Levaditi *et al*, 1932 *d*, D'Aunoy and von Haam, 1936 *b*), the phenomenon of "autosterilizable neuro-infection" may be noted (Levaditi *et al.*, 1933 *a*).

6. *Rats*, by intratesticular and intracutaneous injections (Miyagawa *et al.*, 1936 *a*) White rats have been infected by the cerebral and nasal routes as have kangaroo rats (Eaton, Martin, and Beck, 1942).

7. *Rabbits* can be infected by various routes intracranially (Freund and Reiss, 1931, Coutts *et al.*, 1939, 1942 *a*), intracutaneously (Miyagawa *et al*, 1936 *a*), by corneal scarification, by anterior chamber injection, and by intratesticular injection (Satani and Sano, 1936, Li, 1940 *a*, Coutts, 1946), by intrapulmonary injection (Caminopetros and Photakis, 1935, von Haam and Hartwell, 1937 *a*), the changes being those of an interstitial pneumonia with proliferation of endothelioid cells, subcutaneously and intraperitoneally (Li, 1940 *a*) Malamos (1937) infected the corneae of 5- to 6-day-old rabbits, granulocorpuscles being found. The nasal route is not effective (Schoen, 1939 *d*).

8 *Voles* (and rabbits) may harbor the virus in their brains after intracerebral injection without any apparent symptoms (Findlay, 1933–4).

9 *Fowls*, by intracutaneous injection (Miyagawa *et al*, 1936 *a*).

10. *Squirrels*, by the intracerebral, intratesticular, and intracutaneous routes (Miyagawa *et al*, 1936 *a*, Satani and Sano, 1936).

11 *Marmots*, by intracerebral injection (Malamos, 1937, Nauck and Malamos, 1937)

12 *Sheep*, by intracerebral injection (D'Aunoy and von Haam, 1936 *b*).

13. *Hamsters* have been infected by the nasal and cerebral routes (Eaton, Martin, and Beck, 1942, Beck, Eaton, and O'Donnell, 1944).

14 *Cotton rats* have been infected nasally (Beck, Eaton, and O'Donnell, 1944)

15 Infection could not be passed in the bat by the cerebral route (Meneghini, 1940)

TOXIC PROPERTIES OF THE VIRUS

A labile toxin, not readily separated from EB's, has been demonstrated in heavily infected yolk sac material. After intravenous or less commonly intraperitoneal injection, mice usually die in 4–24 hours, and show necrotic foci in the liver (Rake and Jones, 1943, 1944). The toxin can be converted to toxoid with formalin (Rake and Jones, 1944).

Antibodies can be produced in rabbits or chickens against the toxoid or toxin (Rake and Jones, 1943, 1944). Toxin may be neutralized by convalescent serum (Shaffer *et al.*, 1944); it is not affected by sulfonamides (Rake and Hamre, 1944).

MORPHOLOGY OF THE VIRUS

Elementary and Inclusion Bodies

There is abundant evidence that the small granulocorpuscles, first fully described by Miyagawa, represent the causal agent of LGI. These granules can be found lying free in human and animal exudate and tissue, and characteristically in the interior of mononuclear and histiocytic cells; they measure about 0.3μ in diameter. These bodies were found by Miyagawa in many sites, for example, human, monkey, mouse, and other animal tissues, in tissue cultures, in the chorio-allantois; the presence of the bodies was shown to parallel infectivity in filtration experiments (Miyagawa *et al.*, 1935 *a, b, c, d*, 1936 *a, b*).

Independent proof of the etiological rôle of Miyagawa's granulocorpuscles has been furnished by many workers (Coles, 1936; Satani and Sano, 1936; Tasaki, 1936 *a*; Herzberg and Kohlmuller, 1937; Hoffman, 1937; Nauck and Malamos, 1937; Malamos, 1937, 1938; Caminopetros, 1938 *a*; Cottini, 1938 *a, b*, 1939 *a, b*; Melczer, 1938; Schoen, 1938 *a*, 1939 *a, c*, 1940 *b*; Gavrilof and Fester, 1939; Melczer and Dósa, 1940; Benedek and Olkon, 1941; Levaditi, 1944 *a, b, c*; and by Coutts, see below).

The appearance of the bodies is not unlike that of *Rickettsiae*, and they have been said to develop in the intestine of lice infected with pus (D'Ignazio and Codeleoncini, 1945). There is, however, no justification for classifying Miyagawa's bodies with the *Rickettsiae* (see, e.g., Nauck, 1940).

The Gamna-Favre inclusions are probably derived from the nuclei of leukocytes ingested by macrophages (Li, 1940 *b*; see also p. 773). Favre (1940 *a, b*) has pointed out that his original preparations also contained small granules similar to the Miyagawa granulocorpuscles.

Ishimitsu (1936, 1937) described large eosinophilic inclusions in histiocytes, epithelial and glial cells of mouse brain, and in human lesions. The precise nature of these bodies is uncertain, but they resemble closely the "Russell body-like" inclusions described by Coutts, and believed by him to be viral in origin (see below).

Developmental Cycle

1. *In animals.* Findlay, Mackenzie, and MacCallum (1938 *a, b*) have postulated the existence of a definite life-cycle, analogous to that of psittacosis. Their experiments were performed on mice inoculated intracerebrally. Brains were removed and smears made at intervals from 2 hours onwards. Two main types of body were found, small and large, with the following characters:

The small forms, or elementary bodies, occur as granules intracellularly, or lying free. When inside polymorphs or mononuclears, the bodies are either diffuse, or, more usually, in small clusters. In some cases these clusters become large, and occupy a vacuole in the cytoplasm. This may rupture allowing the cluster of EB's to escape. The bodies found lying free may be diffuse or in clusters. The small forms stain bluish-gray by Castañeda's method.

The large forms are most numerous within 24 hours of inoculation, are three to four times the size of EB's, and stain reddish-purple by Castañeda. They are

arranged in a variety of ways (*a*) they may be found as compact "morula-like" ... hundreds of elementary bodies. (*b*) they may occur in groups
ion in preparations made
er inoculation, large and
lying free.
the smaller, dumb-bell
forms were seen, and also structures of intermediate size, large bodies were seen with short chains of smaller granules attached Findlay visualized the state of affairs as follows After injection the elementary bodies are too scanty to be seen for a few hours, then from the smaller forms which are injected appear large forms. These large forms now divide to form a compact mass, probably inside cells Large forms break away and penetrate fresh cells, to divide up with the production of elementary bodies Eventually only the small forms can be found, the larger "parent" bodies being no longer visible.

Somewhat similar forms have been observed by Gavrilof and Fester (1939) and Coutts (see below).

(2) *In tissue cultures* Similar developmental forms have been observed in tissue cultures (Malamos, 1938, Manabe, 1939 *a*, *b*); homogeneous inclusions in the early stages were observed to become vacuoles containing numerous EB's.

(3) *In eggs* The development cycle can be readily observed in infected yolk sacs (Rake and Jones, 1942; Shaffer *et al*, 1944, Coutts, see below) Rake and Jones found that following the disappearance of the inoculated virus bodies, new forms appeared in yolk sac cells 10–12 hours after the inoculation. These "initial bodies" divided into coccal forms and became arranged within the limiting membrane of a vesicle By 20 hours there was a further increase in size, accompanied by a considerable increase in infectivity; definite EB's were found within large green plaques, or lying free in larger masses The plaques seemed to be produced by enlargement of the larger bodies, and several might appear in one vesicle The EB's escaped to invade fresh cells 28–30 hours after inoculation

(4) *Observations of Coutts* Coutts and his collaborators have studied the morphology of LGI virus for many years, and have made numerous observations on its developmental phases in man (see above) and experimentally (see, e g, Coutts *et al*, 1939, 1942 *a*, *b*, 1943, Coutts, Lerner, and Said, 1941–2, Coutts, Luque, and Hewitt, 1940–1) For the substance of the following paragraphs, and for Figures 38 and 39, we are indebted to Dr. W. E. Coutts of Santiago who kindly sent us a personal communication (1946).

In the early stages of infection in mice inoculated intracranially with egg-cultured virus, inclusion bodies, 0 2–0 5 μ in diameter, can be observed in brain substance, lying free, or in cells. Occasionally these bodies mass together and form clusters Larger forms may appear in plaques, some of which disintegrate and

above)

Observations have also been made in yolk sacs The best results are obtained with histological material fixed in Bouin or Dubosq-Brasil and stained by Mann's method Virus bodies are difficult to find before 24 hours After this time, plaques 4–5 μ in diameter can be found lying free. Some plaques disintegrate and discharge elementary bodies to enter new cells. Occasionally larger units of 0 5–2 μ in pairs or clusters can be observed lying free.

Electron Micrographs

The EB's are similar to those of psittacosis and pneumonitis They are spherical with a dense somewhat irregular central area, and measure 438 ± 47 mμ (Kurotchkin *et al*, 1947)

Filtration

By means of filtration experiments through Gradocol membranes the : virus has been estimated to lie between 0 125 μ and 0.175 μ (Broom an 1936), and 0 1 μ and 0.14 μ (Levaditi *et al*, 1936 *a*), thus confirming observations of Miyagawa *et al.* (1935 *c*).

The virus passes through Chamberland L_2 and L_3, Berkefeld V and N, EK filters The filtrability through Chamberland and Berkefeld filters shown in 1931 independently by Levaditi *et al.* (*a, b*) and by Hellers Wassén.

OTHER PROPERTIES OF THE VIRUS

Cultivation

Tissue culture. Although Voet (1935) could not demonstrate grow other workers have obtained evidence of proliferation in media of the M Li and Rivers type (Meyer and Anders, 1932; Tamura, 1934, 1935; D'Au 1935, *Miyagawa et al, 1936 b; Gavrilof and Fester, 1939; Manabe, 1939* and Leone, 1940 *a, b*). Some of these workers have also studied the develo granulocorpuscles in slide cultures. More recently, growth has been ol embryonic tissues in serum or ultrafiltrate (Sanders, 1940, Curth, C *Sanders, 1940), and by the roller tube technique (Gey and Bang, 1939).*

Eggs. The virus can be propagated on the chorio-allantois and produc matory changes, granulocorpuscles can be found (Miyagawa *et al.*, 1935 and Malamos, 1937; Malamos, 1937; Howard and Hull, 1941, Melczer a 1941).

Although membrane suspensions contain virus, they do not make suit gens for the Frei test (Howard and Hull, 1941).

The virus grows extremely readily in the yolk sac, and produces num clusions in the entoderm, that can be readily separated and purified by sh centrifugation, the embryo is usually killed, even with inocula diluted be a preparation of purified and phenolized EB's, known as "lygranum" is e used for the Frei and complement fixation tests (Grace, Rake, and Shaf McKee, Rake, and Shaffer, 1940, Rake, McKee, and Shaffer, 1940, Shaffer, I McKee, 1940; Rake and Jones, 1942, Shaffer, Rake, and Grace, 1942, Shaf 1944)

Reaction to Physical and Chemical Agents

Cold At 4° C., material should remain active for 3 weeks, and at –1 –20° C. for over a month.

When frozen and dried *in vacuo* over sulfuric acid, infected brain i retain its activity for over 3 months (Findlay, 1933–4), although Miyaga (1935 *e*) stated that infectivity was lost after 30 to 35 days.

Heat. At temperatures from room temperature up to 30° C, the virus mains active for 24 hours, it is killed at 56° C in 10 minutes.

Glycerol. The virus only retains its activity for from 7 to 14 days (1933–4)

Storage is best carried out, therefore, by placing material in the refrig it is to be used within the next few days. To keep virus alive for several it is best to freeze and dry *in vacuo* Durieux (1945), for example, found survive for about 2 years *Infectivity is well preserved in the dry ice box.*

Chemicals The virus is destroyed by formalin (1/1,000), and attenu phenol and sodium ricinoleate (1 1,000) (D'Aunoy and von Haam, 1936 destroyed by 2 per cent urea (Nigg, 1942, Nigg, Hilleman, and Bowser *but not by bile* (Sterzi, 1939 *a*).

Radiation The virus is destroyed by the photodynamic action of methylene blue, and by ultraviolet light (D'Aunoy and von Haam, 1936 *b*)

Association with Other Infections

It has been reported (Vieuchange, 1936) that the association *in vivo* or *in vitro* of poliomyelitis and LGI viruses does not prevent the subsequent development of the typical LGI reaction in mice, nor does it allow poliomyelitis virus to become adapted to the mouse.

Rabies virus and LGI could be passed together cerebrally in mice for many passages (Levaditi, 1942 *a*) Symbiosis was also possible with *Sp duttoni* (Levaditi and Noury, 1944 *b*). Louping ill and LGI viruses could not be passed together (Levaditi, 1942 *b*) On attempting to passage Theiler's virus and LGI intracerebraly in mice, the former rapidly overgrew the latter (Levaditi and Noury, 1944 *c*) When LGI and neurotropic foot and mouth virus were passed cerebrally in mice, the former agent disappeared (Levaditi and Noury, 1943)

Localization in Tumors

When mice were injected intracerebrally with virus, and with a sarcoma subcutaneously, virus was found in considerable quantity in the tumor (Schoen, 1937 *a*, 1938 *b*). Granulocorpuscles could also be found (Schoen, 1938 *c*, 1939 *a*). Virus was also found to localize in spontaneous tumors (Schoen, 1937 *b*)

Chemotherapy

Drugs of the sulfonamide series have been shown to exert a beneficial effect on buboes in guinea-pigs (Levaditi, 1938 *a*, *b*).

More work has been done with mice, and it has been definitely shown that sulfonamides, given orally or by injection, exert a prophylactic or therapeutic effect in mice infected intracranially or nasally (Bar, 1938, Levaditi, 1938 *b*, *c*; MacCallum and Findlay, 1938, Schlossberger and Bar, 1939, Findlay, 1940 *a*, *b*, Jones, Rake, and McKee, 1941, Levaditi and Perault, 1942, Rake, Jones, and Nigg, 1942, Andrewes, King, and van den Ende, 1943, Callomon and Brown, 1943, Rodaniche, 1943, Seeler, Graessle, and Dusenbery, 1943, van den Ende and Lush, 1943, Levaditi, Mentzer, and Noury, 1944; Jones, Rake, and Stearns, 1945; Lépine and Pavilanis, 1947). Animals apparently cured usually harbor virus in the brain or lungs. The carrier rate can be reduced by a course repeated after some weeks (Jones, Rake, and Stearns, 1945). Injected mice treated with sulfonamides still acquire resistance to reinoculation of active virus (Rodaniche, 1942) Wall (1947) found good correlation between complement fixing titers and the duration of treatment; mice treated early and long developed scarcely any antibody, those given treatment late always developed antibodies Virus could always be isolated from treated mice, but less readily after a prolonged course.

In vitro, contact of sulfonamides and virus reduces virulence when subsequently tested in mice, but is not virucidal (Holder, Levine, and Bullowa, 1942).

Andrewes, King, and van den Ende (1943) tested a large number of other chemotherapeutic drugs, but found none effective

Penicillin is toxic to mice infected with LGI intracerebrally, but it exerts some protective effect (Levaditi and Vaisman, 1945, Rake and Jones, 1946).

Chemotherapeutic experiments have also been carried out in the yolk sac of the fertile egg Sodium sulfadiazine given immediately after the injection of virus delayed death of the embryo, penicillin given up to 96 hours later did the same (Meiklejohn, Wagner, and Beveridge, 1946). Hamre and Rake (1947) found that streptomycin and streptothricin had no effect *in vitro* or in the yolk sac on the virus. They confirmed the activity of penicillin, but found that it decreased as the drug was used in increasing purity.

Relationship to Other Viruses

The interrelationship of the LGI–psittacosis–pneumonitis group is discussed in Ch. LXVII.

The virus of Durand's disease is unrelated to LGI virus (Findlay, 1942).

IMMUNITY REACTIONS

Active Immunity

Animals become resistant, even to intracerebral injection, on recovery from infection. According to Schoen (1939 *b*) no histological changes develop after the challenge dose.

In man, it seems to be doubtful whether a complete cure of LGI often results, for the Frei and CF tests frequently remain positive. This is most readily explained by the continued presence of the virus, if not in an active condition then latently. Virus was isolated from a lymph node 4 months after a full course of sulfadiazine (Heyman, Wall, and Beeson, 1947). However, in certain cases the tests do become negative, it seems reasonable to conclude that primary and secondary cases may become free of infection, although this is unlikely to occur with the later manifestations. Brandt (1941) suggests that a considerable percentage of cases heals spontaneously, especially if buboes develop.

Frei's Test

Antigens.

Human material. In the original Frei technique (1925) the antigen is prepared by diluting pus from a known case of LGI with 5 times its volume of normal saline The material is heated for 2 hours at 60° C. and on the following day for another hour As a control, heated saline is used In the test, 0 1 c.c is inoculated in the forearm It is convenient to dry pus *in vacuo* from the frozen state, and reconstitute as required (Grace, 1934). Filtrates of pus prove inactive (Paulson, 1936).

Antigen can be prepared from the feces of cases of colitis due to LGI (Paulson, 1937, 1938, 1939, Paulson and Kravetz, 1938).

CSF has been used as a source of antigen (Chevallier and Bernard, 1932, Midana and Vercellino, 1934, Koschucharoff, 1938) Ottolina (1941, 1943) has recorded the occurrence of vesicle formation when CSF is used as antigen

Reiss (1934, 1935) postulated the presence of an antigen in the serum of early cases, and claimed that such serum could be used as Frei antigen, mixed with the usual bubo pus antigen, an enhanced lesion resulted Le Gac (1936) obtained similar results, but Haynes (1935) and Howard and Strauss (1936) could not confirm these observations

Animal tissues Infected animal tissues have been recommended, and are of some value Unfortunately there is a definite tendency for nonspecific reactions to occur with the control Also, injection of animal tissue may render the patient sensitive to a further test with the same material (Jansen, 1938)

Monkey brain has been used (von Haam and Lichtenstein, 1935). Durieux (1945) recommends the use of brain desiccated *in vacuo* in individual ampules

Mouse brain has been widely employed as antigen It can be desiccated *in vacuo*, and made up into antigen as required, with saline (Grace, 1934). Mouse brain tends to produce nonspecific reactions in normal people, and chiefly for this reason has been found unsatisfactory by certain workers (e g, Strauss and Howard, 1936, Binkley and Love, 1938) Others, however, have found it satisfactory, if a potent antigen giving a papule measuring 7–10 mm is used (D'Aunoy and von Haam, 1936 *a*, Grace and Suskind, 1936 *a*, *b*, von Haam and Hartwell, 1937 *b*, Reider and Cañizares, 1938, Grace, 1939, Costello and Cohen, 1941)

Rabbit lung antigen was recommended by Caminopetros and Photakis (1935), but not by von Haam and Hartwell (1937 *a*) Guinea-pig gland was used by Grace and Suskind (1940).

Cultured virus The culture fluid in Maitland's medium has been used as antigen (Tamura, 1934), also the fluid from roller tube cultures (Gey and Bang, 1939).

Egg virus. Lygranum antigen was first introduced by a group of American workers (Grace, Rake, and Shaffer, 1940, Shaffer, Rake, and Grace, 1942, and see above) It is
the EB's by centrifugation, and
l eggs is used as control This
nd its value has been confirmed
ulkin *et al*, 1941, Axelrod, 1942,
obinson, 1942, Curth, 1943, Gel-
1943; Smadel,

han to mouse
occur, due to
ygranum is as
vided a papule
measuring 7 mm. is regarded as the minimal size of a positive reaction

A saline suspension prepared from yolk sacs heavily infected and inactivated with 2 per cent. urea or 0 5 per cent. phenol has been found satisfactory (Nigg, Grace, and Hilleman, 1947).

Reaction to Frei's Test

In cases of LGI, following the inoculation of antigen, an area of erythema results with central papulation The test should be read after 48 hours It is generally believed that in true positive reactions the papule measures 7 mm or more. Unfortunately, doubtful reactions up to 4–5 mm in diameter may occur in uninfected persons, or may be given by the control material as well.

The reaction becomes positive 2–6 weeks after onset in a very high percentage of cases, false positives, if a sizeable papule is produced, are rare (Frei, 1932 *a*, *b*, 1933, de Wolf and van Cleve, 1932, Jersild, 1933, Bacon, 1935, Lebeuf, 1939). Negative results in definite cases are unusual, and are usually due to factors tending to reduce dermal sensitivity

The intradermal Frei test may cause an exacerbation of the disease, but serious reactions are very rare A most unusual case was reported by Keim and Wakefield (1939), where flaccid paralysis developed 11 days after the use of a mouse brain antigen injected intradermally.

It is probable that despite apparent cure, even with sulfonamides, the Frei test remains permanently positive (David and Loring, 1936, Grace, 1941 *a*, Heyman,

and Monetta, 1938, Michelson *et al*, 1938, Monetta and Coutts, 1941, Axelrod, 1942).

Histological appearance.

The papule in a positive case shows cellular infiltration and necrosis (Kornblith, 1936) Smith (1940) has reported that after 24 hours the epidermis shows marked edema, spongiosis, and almost vesiculation, the cutis shows edema, and there is an accumulation of lymphocytes and a few polymorphs in the perivascular spaces After 72 hours, there is slight hyperkeratosis and some epidermal edema. In the cutis, there is a noticeable increase in connective tissue cells, the perivascular spaces show a definite infiltration with lymphocytes The appearances after 5 days are similar, with less epidermal edema and spongiosis

The nature of the intradermal Frei test.

It is presumed that the Frei test is a sensitivity phenomenon dependent on the presence of a focus of infection with the virus of LGI Frei antigen does not contain living virus. Seitz filtrates of EB suspensions elicit the reaction, perhaps due

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Cultured virus The culture fluid in Maitland's medium has been used as antige (Tamura, 1934), also the fluid from roller tube cultures (Gey and Bang, 1939).

Nigg, Hilleman, and Bowser (1946) found that antigenicity was enhanced by phenolizing yolk sac suspensions at once and incubating at 37° C for 22–60 days. Boiling induced the same degree of enhancement. Autoclaving phenolized suspensions also induced enhancement. They concluded that the mechanism of enhancement by heat or phenol was the same. Perhaps these agents dissociate a protein-polysaccharide-lipoid complex responsible for CF, and the greater dispersion of the antigen accounts for the enhancement. Alternatively an inhibiting factor may be inactivated.

Experimental.

Complement fixing antibodies develop in the sera of rabbits, mice, hamsters, and other rodents immunized with inactivated or active virus (Hildebrand, 1936; Eaton, Martin, and Beck, 1942) In mice that recover, antibodies can be found two weeks after inoculation (Wall, 1947).

In man.

A number of workers has detected complement fixing antibodies in convalescent serum using Frei antigen, or gland antigen (Hecht, 1935, Coutts and Ponce,

man and Webb, 1946, Shaffer and Rake, 1947).

Bowser and Nigg (1946) found that the majority of sera from cases of primary and secondary syphilis reacted in CF tests with specific and control boiled phenolized yolk sac antigens, nonspecific reactions appeared to be eliminated by using the supernatant of boiled phenolized antigen.

In CF tests with lygranum, serum is often used diluted ⅕ only. Dulaney and Packer (1947) found that in this dilution nonspecific reactions tended to occur in healthy persons, in persons suffering from upper respiratory infections, and in the presence of other venereal infections They concluded that fixation with a serum

certain cases of syphilis Where there is a double infection, such absorption leaves the reaction with lygranum unaffected.

Grace, Shaffer, and Rake (1943) concluded that the explanation of the majority of unsuspected positive reactions was the existence of latent LGI This is confirmed by the fact the sera of cases of congenital syphilis rarely fix complement with lygranum (Shaffer and Rake, 1947). Prolonged sulfonamide therapy may not remove CF antibodies, suggesting the continuance of infection (Grace and Rake, 1943)

The children of Frei-positive mothers show complement fixing antibodies in the first 2–3 months of life, but these disappear thereafter (Levine, Bullowa, and Scheinblum, 1943, Beeson and Miller, 1944 *b*).

to the presence of a soluble antigen (Rake *et al.*, 1941 *b*). The pus in the center of a Frei reaction still contains antigen in an active form (Strauss and Howard, 1934). Injection of serum of Frei-positive cases into healthy persons causes them to react positively, the Prausnitz-Kustner technique can be used to demonstrate transmission of sensitivity (Melczer and Sipos, 1938 *a*, Marks, 1942). It has been stated that convalescent serum neutralizes the activity of the Frei antigen (Gottlieb, 1932 *a*, Tasaki, 1937 *a*); but others have failed to confirm this observation (Calatayud, 1933, Haynes, 1935).

The reverse Frei test.

Antigen can be prepared from the suspected case and injected into a known case (Wiedmann, 1935).

The intravenous Frei test.

Following intravenous injection of Frei's antigen, pyrexia develops in a few hours, and this may be of diagnostic significance (Tasaki, 1937 *b*). Mouse brain antigen can be used (Decker, Cañizares, and Reider, 1939, Costello and Cohen, 1941; Morris and Cañizares, 1942).

Virus Neutralizing Antibodies

Experimental.

Virus neutralizing antibodies can be produced in rabbits inoculated with living or formolized virus, but only with difficulty (Okanishi, 1937, van den Ende and Lush, 1943). Mice convalescent from infection develop neutralizing antibodies (van den Ende and Lush) These workers titrated antibody by inoculating mixtures of a pneumotropic strain and serum nasally in mice, the number of foci of consolidation being counted

Convalescent serum was found not to protect against experimental infection in guinea-pigs (Caminopetros, 1939).

In man.

Although Hildebrand (1936) could not demonstrate neutralizing properties in human convalescent serum, other workers have detected these antibodies (Levaditi *et al*, 1932 *a*, *c*, *e*, Miyagawa *et al*, 1935 *e*, Wassén, 1935, Tasaki, 1937 *a*, Caminopetros, 1938 *b*, Gey and Bang, 1939, Rodaniche, 1940, Menk and Mohr, 1941, Frei, 1943; Grace, Shaffer, and Rake, 1943)

Rodaniche used a mouse brain virus, and injected mixtures intracerebrally

Complement Fixation

Nature of the reaction.

Complement fixation can be demonstrated between convalescent or hyperimmune sera and various types of LGI antigen Thus infected lung or gland tissue has been used (see below). Fixation can also be demonstrated between sera and the Frei reagent prepared from pus Infected yolk sac material (lygranum) in the form of an untreated suspension, will fix complement, purified EB's also fix complement. Further, there is a soluble complement fixing antigen in yolk sac suspension (which also elicits the Frei reaction), for filtrates or supernates are active (Rake *et al*, 1941 *b*, Nigg and Bowser, 1943) Part of the complement fixing antigenic material is resistant to boiling (Nigg and Bowser, 1943)

The CF properties of infected yolk sac material can be enhanced in various ways. Thus, treatment of a crude suspension with phenol, urea, or ether yields a better antigen than is afforded by EB's (Nigg, 1942, Nigg and Bowser, 1943, Hilleman and Nigg, 1946)

BIOLOGICAL PRODUCTS IN THE TREATMENT OF LGI

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blith, 1939). Caminopetros (1939) recommended the injection of antigen into the bubo and rectal tissue and claimed more rapid healing Tamura (1935) suggested the use of heated culture virus. Jonesco-Mihaiesti *et al.* (1934) recommended a heated vaccine prepared from the organs of a monkey injected intraperitoneally. Filtrates of ground infected glands have been used by Zahawi and Akrawi (1940).

Pyrexia produced by TAB or Dmelcos vaccines is said to be beneficial (Batchelor, 1937).

Numerous workers have found the sulfonamide drugs to be effective, apparently even in the later stages. As the Frei test may remain positive after treatment, it is doubtful whether the infection is entirely eliminated (Earle, 1939, Hebb, Sullivan, and Felton, 1939, Midana, 1940; Brandt and Greenblatt, 1941, Schamberg, 1941, Stammers and Law, 1942, Pico Estrada, Lima, and Calcagho, 1945, Wall, 1946).

Normally sulfonamide destroys the infectivity of virus in buboes in 2–3 weeks, in one case, however, virus was isolated 4 months after a full course (Heyman, Wall and Beeson, 1947).

Penicillin has been reported on favorably, used locally or by injection (Willcox, 1946, Mayne and Bain, 1947).

Antimony appears also to be useful.

Human convalescent serum was found effective by Videla and Capulo (1939), but animal serum proved too toxic (Sagher, 1944). Caminopetros (1939) did not find human serum to be of value.

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LABORATORY DIAGNOSIS

(*a*) *Routine bacteriological examination* Films of pus should be thoroughly examined for organisms, including Ducrey's and tubercle bacilli. Cultures should be prepared aerobically and anaerobically, not omitting the special rabbit blood technique for Ducrey's bacillus as used by Nicolle (1923). Supposing no organisms are found by this examination, one is fully justified in proceeding to special diagnostic measures.

(*b*) *Frei's test should be carried out with lygranum or other antigen, and suitable control material.* If desired, antigen can be prepared from mouse brain infected with the patient's material and used in known cases of LGI (Frei, 1943). It may be necessary to carry out the reverse Frei test, i e., preparing antigen from the pus of the suspected case, and injecting it intradermally in a known case.

(*c*) *Animal experiments* Monkeys and mice are injected intracerebrally, and guinea-pigs in the groin with pus, at least 4 guinea-pigs and 6 mice being injected. *It should be remembered that the rhesus monkey is not nearly so susceptible as the* green or callithrix monkey. Pus for inoculation should be diluted 1/10–1/20 in saline, and shaken mechanically with glass beads, lymph nodes should be ground and lightly centrifuged (Wall, 1946) Saline can be inoculated into buboes or lymph nodes, and aspirated (Heyman, Wall, and Beeson, 1947).

Coutts (1946) recommends the inoculation of pus, or the implantation of tissue in the anterior chamber of the rabbit's eye. After 3–5 days the contents are inoculated in the yolk sac.

(*d*) *Inoculation of eggs and tissue cultures* Virus can be isolated by direct inoculation of bubo material in the yolk sac (Shaffer, Rake, and McKee, 1940, Shaffer *et al.*, 1944) If no EB's are seen at the primary inoculation, 4–5 "blind" passages should be carried out (Wall, 1946) Virus may also be isolated by direct inoculation in tissue cultures (Sanders, 1940)

(*e*) *Serological tests* A complement fixation test should be carried out with lygranum antigen Wassén (1935) and Frei (1943) have used virus neutralization tests Antigen from the patient can be tested with animal antiserum to see if complement is fixed (Frei, 1943).

(*f*) *Histological examination* Coutts (1946) recommends that smears, e g, of *bubo pus, are fixed in Bouin or Dubosq-Brasil, and stained with 3 per cent.* magenta or 2 per cent mercurochrome with nigrosin background, or by Mann's stain. For sections, the same fixatives should be used Mann's or Machiavello's stains give the best results, stained by Mann's method, inclusions stain indigo or purplish-blue.

(*g*) *Other tests* The blood should be examined by count and biochemically.

(*h*) To exclude syphilis, the Wassermann and flocculation tests should be performed, and to exclude ulcus molle (soft sore, chancroid) 0 2 c c. of Ducrey's bacillus vaccine should be injected intradermally A positive reaction to this Reenstierna's test is indicated by the formation of a wheal and erythematous halo in 24 to 48 hours, perhaps with a rise of temperature up to 102° F This reaction denotes the existence, past or present, of a Ducrey infection (see, e.g, Lees, 1937) It is not *at all easy, however, to separate LGI and Ducrey's infection* There is no doubt that a considerable number of colored persons are infected at the same time with these diseases, and probably with syphilis also (Brandt and Torpin, 1940, Robinson, 1940, Young, 1944)

the coexistence *of positive skin* tests t co-reactions can occur in the acute ur in soft sore, positive CF tests for LGI may also occur (Brandt and Torpin, 1940, Heyman, 1946).

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head, 30 days, upper extremity, 50 days, and leg, 44 days (see also Dodero, 1937 *b*, Jordan, 1939, Boecker, 1940).

Livon and Placidi (1929) reported a case developing 13 days after a face bite (see also Wright and Gosden, 1946). Proca and Bobes (1940) found that short incubation periods were more frequent in Bucharest than elsewhere, and that short incubations were more frequent in children under 15 years than in older persons.

Various cases developing after a prolonged incubation period have been reported: e g, after 9 months (Jagot, 1886), after 1 year, although antirabies treatment was administered following the bite (Rochaix, 1924, Stuart and Krikorian, 1932 *b*), after 14 months (Hajare, 1933), after over 3 years and 2 months, although antirabies treatment was administered (Iyengar, 1935).

Certain street viruses have properties very similar to those of *virus fixe*, including a short incubation period in animals It was suggested by observers in Tonkin that the reason why the local incubation period was short (under 4 weeks), and antirabies treatment often failed, was because of the prevalence of these virulent *renforcé* strains (Bablet and Joyeux, 1930, Genevray and Dodero, 1934; see also Dodero, 1938 *b*) However, this concept does not seem to be generally accepted.

Manifestations of Human Rabies

Although furious rabies is the commonest manifestation, other types may occur. It is not proposed to give the clinical features of classical rabies in great detail, as these are well known. Attention will be concentrated on certain aspects of the disease less well recognized

1. Furious rabies.

Prodromal symptoms The patient frequently feels pain at the site of the wound, is restless, suffers from headache and complains of aching in the back and limbs

Course of the disease. The characteristic symptoms of hydrophobia soon develop At first, the hydrophobic attacks occur only on attempts being made to drink, when the head is thrown back, the eyes are fixed, and the hands may go into spasm. The patient has a dread of water and shudders at the very sound of the word Later, the spasms are more readily provoked by drafts or loud sounds, and swallowing becomes almost impossible The patient is very dyspneic, especially on inspiration, and the respiration rate is rapid, while Cheyne-Stokes rhythm may occur The patient usually expectorates freely and saliva is ejaculated in all directions, sneezing may be a prominent feature With the progress of the infection the patient becomes noisy, restless, and often actively "furious," breaking articles of furniture and even attacking bystanders. Attempts may be made to bite. The mind is lucid between the attacks, but as these become more frequent the mental derangement increases and hallucinations and delusions may be experienced (Figs 40A and B). Aerophobia can be demonstrated by waving a fan before the face.

After a leg bite the first symptoms may be referable to the lumbar cord, such as dysuria and disorders of defecation After an ear bite cerebellar symptoms have been recorded. The temperature is usually raised, especially before death, and may continue to rise thereafter (see, e g, Gamaleia, 1887 *a*, Schaffer, 1890). The pulse is usually increased in rate The pupils are usually dilated but may contract and dilate alternately. The reflexes are increased at first, but later become absent. If the patient lives long enough, paralysis of the limbs usually occurs as a terminal event. Newborn children of rabid mothers seldom, if ever, contract the infection (see, e g, Genevray and Dodero, 1935) Blatt *et al* (1938) have published some informative pictures of rabies cases

2. Spinal manifestations.

(*a*) *The landriform type* This type of rapidly ascending and usually fatal paralysis has been recorded from time to time in cases of untreated rabies (e g,

CHAPTER LXXIII

RABIES (HYDROPHOBIA) IN MAN

Rabies is a disease of great antiquity; it was known in the time of Aristotle, Pliny, and Celsus, and has remained endemic in various parts of the world to this day. The disease is primarily one of animals—mainly dogs, cats, herbivora, and certain carnivora; man is usually infected by a bite of a rabid animal Great interest has been taken in a form of rabies in Trinidad, the transmitting agent being a bat.

Little progress was made in the isolation of the etiological agent of rabies until, towards the end of last century, it was found that animals, especially dogs and rabbits, could be infected with human nervous tissue. The next step was Pasteur's claim that dogs could be actively immunized with cords of attenuated virulence. Later, he applied this discovery to the treatment of bitten persons. The etiological agent of rabies is now well recognized to be a filtrable virus. Despite over 40 years' research, however, the precise significance of the characteristic inclusion body—the Negri body—is still uncertain.

With regard to the literature, this has reached vast proportions, and particular mention should be made of the excellent reviews published twice yearly from 1924–43 by A. G. McKendrick, and since then by I A. Galloway in the *Tropical Diseases Bulletin*. Schweinburg (1937)[1] has published a very lengthly review of modern work. A prominent part in experimental study has been played by MM. Remlinger and Bailly of Tangier, and many of their researches have been collected together (1938 *a*).

CLINICAL FEATURES

Factors Predisposing to Rabies

There has been some discussion as to whether certain factors may predispose to rabies, or modify the resultant type of the disease Thus, in the days of Gamaleia (1887 *c*) alcohol was definitely blamed as a predisposing cause of rabies, but experimental work (see p. 847) has shown that this is unlikely. The nervous constitution of the bitten person plays a part in determining the type of rabic syndrome —furious or paralytic—which develops Persons infected with virus and given anti-rabies treatment may develop rabies a considerable time later if subjected to an emotional shock (Remlinger, 1946 *b*) On the whole, however, there are no predisposing factors of any great importance

Incubation Period

It has been held that the nearer the bite is to the central nervous system, the shorter is the incubation For example, Webster (1944) gives the following figures for the average incubation periods face, 30 days, arm, 40 days; leg, 60 days Johnson (1943), however, disputes this, and suggests that the amount of virus introduced and the type of tissue involved are the chief determining factors, the incubation period after head and face wounds is usually short because of severe laceration and exposure of sensory nerves. Johnson gives the following average incubation periods

[1] References are appended at the conclusion of Ch LXXIX, p. 887 et seq

(*b*) *Transverse myelitis* Roy (1936) described a case, at first thought to be idiopathic transverse myelitis, which developed definite hydrophobic symptoms shortly afterwards.

3. Paralytic rabies.

The first description of paralytic rabies in man is said to have been that of van Swieten (see Brouardel, 1897) However, until the description of Gamaleia (1887 *a*, *c*, *e*) no form of human rabies other than the hydrophobic type was generally recognized Since that date other similar cases have been recorded (see e g, Van Gehuchten, 1907, Koch, 1912, Babès, 1912 *a*, Marie and Chatelin, 1919, van Wulffren Palthe, 1930, Love, 1944). The usual symptoms presented by such cases are pyrexial disturbance, paralysis of the limbs, paraplegia, paralysis of the face and eye muscles resulting in ptosis and strabismus, fibrillary tremors, irregular respirations with respiratory spasms, and dyspnea, priapism and ejaculations, sensation is usually preserved, but some cases are anesthetic, pains may occur in the limbs later to be paralyzed. Hydrophobic symptoms may be absent in such cases The histological features of paralytic and furious rabies would appear to be similar and are described on p 797 (see Van Gehuchten, 1907, Higier, 1912, Federoff, 1925–6, Marie and Chatelin, 1919)

The above description applies to paralytic rabies contracted from rabid dogs and other animals, and should not be confused with paralytic rabies occurring in Trinidad, spread by bats.

CLINICAL PATHOLOGY

The blood A number of workers has reported the hematological findings in untreated cases of rabies (e g, Courmont and Lesieur, 1901, Leger, 1923, Jonnesco, Valter, and Teodisiu, 1927, Massias, 1928). Differential counts usually show a relative, and sometimes an absolute, increase in polynuclears Monocytes and eosinophils are usually diminished, and this change appears some 2 days before the onset of symptoms The BSR is said to be increased (Stanesco and Enachesco, 1939)

The cerebrospinal fluid is usually normal, although an increase of leukocytes up to 100 per c mm has been reported (see e g, Lesieur, 1904, Marie and Chatelin, 1919, Wright and Gosden, 1946)

The urine is usually loaded with albumin, and may contain sugar (see, e g, Southam, 1879)

DISTRIBUTION OF STREET VIRUS IN THE BODY

Site of bite The virus has been found in the scar (Pace, 1903), but may not be present in all cases (Sabin and Ruchman, 1940)

Central nervous system In the past it has been stated that virus is found most constantly in the medulla (Gamaleia, 1887 *c*, Pace, 1903), but it is doubtful if this is correct Employing the sensitive mouse inoculation method, Leach and Johnson (1940 *a*) found virus in the anterior central gyrus, thalamus, pons, medulla, and spinal cord of 3 cases, the thalamus titered 10^{-4} in all cases Virus may also be found in Ammon's horn and the olfactory bulbs (Sabin and Ruchman, 1940)

Paltauf (1909) claimed to have found street virus in the brain of persons who died from intercurrent diseases early in antirabies treatment

Virus does not occur in the cerebrospinal fluid

Nerves Pasteur, Chamberland, and Roux (1884) reported that virus could be obtained from the vagus and sciatic nerves of human cases of rabies After a bite on one leg, virus has been found in the corresponding sciatic nerve and frequently in the contralateral nerve as well (Pace, 1903)

Roux (1888 *a*, 1889) reported that he had found virus in the axillary nerves

Remlinger, 1906 *c*; Lemke, 1934; Fife, 1945). It should not be confused with the ascending paralysis which may complicate antirabies treatment as a neuroparalytic accident (see Ch. LXXIX).

Knutti's (1929) case illustrates the symptomatology of acute ascending paralysis. The case was remarkable in that although the virus of rabies was isolated there was



Fig 40 Case of rabies in an Egyptian woman, exhibiting symptoms of hydrophobia. In A, the patient is shown refusing to drink water In B, she has taken a sip of water, and the photograph taken immediately afterward illustrates the facial appearances during a hydrophobic spasm

(Photograph by the authors, reproduced by courtesy of Dr Ibrahim Shahin, Director, Antirabies Institute, Cairo.)

no history of a bite from a rabid dog Apparently, however, the patient had been bitten by a squirrel (Love, 1944) The illness started with some pain in the left leg and lower abdomen The leg soon became numb and spastic, and the patient felt depressed mentally, next, the leg became partly anesthetic and showed a flaccid paralysis The paralysis then spread to the other leg Just before death the hands had become numb also The ganglion cells of the cord, especially in the anterior horn, showed all degrees of degeneration, most of the motor cells having been completely destroyed The nerves arising dorsally showed areas of degeneration with loss of myelin, sometimes this loss was almost complete, only a shell of tissue remaining around an infiltration of mononuclears and phagocytes

The rabic nature of this most interesting case was established by finding Negri bodies in certain anterior horn cells, and by transmitting the infection to rabbits by scarifying the skin with a cord suspension

mal, there the virus presumably invades the terminal nerve filaments Some local pullulation may take place, as virus has been isolated from the bite wound even when scarred (Pace, 1903). The infecting animal does not necessarily need to bite, as cases of rabies developing after clawing and scratching only have been recorded (e g., by Remlinger, 1906 *b*, Kocevaloff, 1923) In other cases it appears that rabies may be contracted by the virus passing through the unbroken skin, although such an occurrence must be regarded as a rarity. Thus, Stuart and Kirkorian (1932 *b*) reported a case following on soiling of the apparently unbroken skin of the hands with the saliva of a rabid calf. Greval (1936) recorded cases following on the licks of rabid dogs (see also Shields, 1939). Glusmann (1928) reported a case of rabies with no history of contact with a rabid animal. We ourselves observed a fatal case in a veterinary surgeon returned from East Africa, who as far as he was aware had not been exposed to infection from a mad dog.

Spread of the virus in the body. Having gained entrance through a wound made by a bite, scratch and claw, or, rarely, through the unbroken skin, it is believed that the virus ascends in the nerve trunks This theory is based on various facts. Thus, Pasteur, Chamberland, and Roux (1884) reported that virus had been isolated from human vagus and sciatic nerves. Roux (1888 *a*, 1889) was able to isolate virus from the axillary nerves, following an arm bite, the virus was absent from the radial nerves, suggesting that it had passed centrally Roux also noted that virus might be found in the nerves of the healthy side, clearly suggesting a general dissemination throughout the peripheral nervous system. Virus was isolated from both sciatic nerves after a bite on one leg only (Pace, 1903)

Additional evidence that spread occurs along nerves is afforded by the fact that, following leg bites, the first part of the central nervous system to be involved is often the lumbar cord, producing symptoms of dysuria and disorders of defecation. Correspondingly, following ear bites, the cerebellum may be the first part of the central nervous system to be affected Further, in cases that have died early, it has sometimes been possible to observe that the main pathological changes in the central nervous system occur in the cord corresponding to the bitten limb Histological changes have been observed in the nerves and spinal ganglia corresponding to the bitten limb (see e g, Marinesco and Draganesco, 1932)

Manouélian (1936 *b*, *d*) has found Negri bodies in the endoneurocytes of the sensory and sympathetic fibers of the face, and in the sympathetic ganglia in cases of rabies This certainly suggests that virus diffuses through the peripheral nervous system (*septinévrite*) in man, as in animals

Despite the above observations, the question is in need of review, using more sensitive methods In particular, the rôle of blood and lymph requires investigation There are evident similarities to the much debated question of the spread of tetanus toxin

PATHOLOGY

1. Central Nervous System

The general neuropathological features of rabies have been described by numerous authors (e g, Benedikt, 1874, Schaffer, 1889, 1890, Fañanás, 1918, 1922, Marie and Chatelin, 1919, Krinitzky, 1926, Lowenberg, 1928, Bassoe and Grinker, 1930, Rojas, 1932, Riesman *et al*, 1933, Verhaart, 1934, Schukru-Aksel, 1934, 1935–6, 1937–8, Tinel, 1938, Rodriguez, 1943, Costa and Netto, 1944) Certain authors give references to earlier workers (see Van Gehuchten and Nelis, 1900 *a*, Federoff, 1925–6)

Nerve cell lesions A number of authors has described changes in the nerve cells of a varied nature (see e g, Bosc, 1903 *a*, Marinesco, 1909, 1910, Marie and Chatelin, 1919, Marinesco and Draganesco, 1932, Riesman *et al*, 1933). Thus the Nissl bodies are often lost, the cells vacuolated, and the nuclei degenerated Cajal's

following an arm bite He also mentioned that the virus might be found in the nerves of the unbitten side Bardach (1888) also isolated virus from a nerve, central to the site of a bite.

Saliva. Suitable inoculation tests have demonstrated the infectivity of the human saliva [illegible] 1881 *a*, Raynaud and Lannelongue, 1881; [illegible] 1931 *a*, 1933, Sulkin and Harford, 1943; [illegible]

Sec[illegible]s, been isolated from the lacrymal, *parotid, sublingual, and submaxillary glands* (Pasteur, Chamberland, and Roux, 1884; Bardach, 1888, Pace, 1903, Tscheschkow, 1931; Leach and Johnson, 1940 *a*).

Urine. Virus has been found in the urine (Jonnesco, 1927 *b*).

Milk. The milk of a lactating woman was found to contain the virus (Bardach, 1887 *b*).

MORTALITY

Once rabic symptoms develop the disease is uniformly fatal. Although death does not usually occur until the disease has lasted for some few days, sudden death *may occur in the early stages, and Bobes (1928) has suggested that this is due to an* affection of the cardiac nerves.

Owing to the large number of variable factors, it is extremely difficult to assess the chances of a completely untreated person dying of rabies from the bite of an infected animal Cornwall (1923) suggested that strains of virus vary in infectivity, and quoted a series of 423 untreated persons bitten by rabid dogs, with 35 per cent. mortality. On the other hand it is known that many persons bitten by rabid dogs and untreated escape infection (see e.g., Breault, 1945), and Cornwall's figure is probably well above the average. It would seem reasonable to suggest that no more than 5–15 per cent. of persons bitten by rabid dogs, and untreated, actually develop rabies, this figure being influenced by various factors

With regard to the severity of the bite, reporting Kasauli deaths, Harvey and McKendrick (1923) found a deep bite to be 4 8 times as serious as a superficial one. They also noted that efficient and timely cauterization lowered the deaths in the proportion of 4 3 and interposition of clothing by 6 6 1, the site of the bite is also of importance, the proportional mortalities according to the site being face 7.7, arm 1.15, and leg 0 7.

Wolf bites are more serious than dog bites, and the mortality in untreated cases is at least 60 per cent.

Further information on mortality will be found in the section on antirabies treatment (see p. 868)

PATHOGENESIS

Experimental It is generally conceded that rabies virus spreads from the portal of entry to the central nervous system by the nerve fibers This view *is largely* based on experimental work (see p 828)

It has been found that experimental animals can be very readily infected by intraneural injection After such inoculation, as after intracranial injection, virus spreads throughout the whole nervous system and can be isolated from the brain, sciatic, median, and other nerves This spread is termed *septinévrite*, and takes a few days. Thus, after intraneural injection virus cannot be demonstrated in the brain or other nerves for 5 to 6 days After intracranial injection virus cannot be found in the peripheral nerves for a similar number of days Experiments undertaken in animals with divided nerve tracts have shown that this operation effectively prevents virus reaching the portion distal to the site of inoculation.

Portal of entry of the virus Virus usually enters the body through the skin, being deposited in the depths of a wound by the infected saliva of the biting ani-

by leukocytes, especially in the anterior horn, perivascular cuffing, general congestion, and hemorrhages are also found, these hemorrhages are said to be most marked *posteriorly*. *The anterior horn cells are usually degenerate, and this process frequently* affects the white fibers also Generally speaking, the inflammatory changes are most marked in the gray matter. The portion of the cord most involved, both naked-eye and microscopically, is often that corresponding to the bitten limb, i e., the lumbar cord in leg bites and the cervical cord in arm bites *(see, e g, Schaffer,* 1889)

Midbrain Cellular infiltration and hemorrhage usually occur in the pons and *basal nuclei In certain cases the substantia nigra and neighboring nuclei may be* markedly involved in the inflammatory reaction. Thus, Slotwer (1926) gives the following sites where cellular changes may be marked substantia nigra, "supraoptic" nucleus, paraventricular nucleus, grisea centralis, and corpus luysi (see also Lowenberg, 1928) In the substantia nigra there is often marked neuronophagia of the pigmented cells

Cerebellum Changes in the cerebellum may occur, especially after bites on the *ear (see Gamaleia, 1887 a, Marie and Chatelin, 1919). The Purkinje cells may* be destroyed, and undergo neuronophagia.

Cerebrum Beyond some congestion, and the occasional presence of Negri bodies or degenerate neurons, there is little to be seen

Differential diagnosis Various authors have likened the lesions of rabies to those of epidemic encephalitis (see, e g, Klarfeld, 1922, Schukru-Aksel and Spatz, 1925, Lowenberg, 1928, Bassoe and Grinker, 1930, Rojas, 1932). This likeness is most evident when the lesions are prominent in the substantia nigra and neighboring areas. The presence of Negri bodies, of course, quite rules out the diagnosis of epidemic encephalitis.

2. Other Parts of the Nervous System

Sympathetic ganglia Chachina (1926) reported that cellular infiltration, especially around vessels, and degeneration of neurons might be found (see also Marinesco and Draganesco, 1932)

Peripheral nerves Changes in the nerves connected with the site of the bite take the form of congestion and cellular infiltration, even at a distance from the wound *(see Marinesco and Draganesco, 1932) Giant cells may be found in the nerve (see,* e g, Nepveu, 1872, Dawydowski and Dwijkoff, 1928) Manouélian (1936 *b, d*) has reported that the sensory nerve filaments of the trigeminal in the face may show characteristic lesions Thus, Negri bodies occur in the nerve cells (endoneurocytes) which are found at intervals in the nerves Further, these cells may show degenerative changes and Van Gehuchten and Nelis lesions.

3. The Remainder of the Body

Salivary glands Changes in the submaxillary, parotid, and other glands have been reported (see Nepveu, 1872, Babès and Jonnesco, 1909 *a*) The acini are usually dilated and filled *with granular debris* The vessels and ducts are surrounded by a cellular infiltration consisting of fibroblasts, lymphocytes, and plasma cells

Kidneys The kidneys may show hyperemia and the tubules cloudy swelling and epithelial desquamation (Babès and Jonnesco, *1909 c*)

Adrenals The medulla may be infiltrated with mononuclears, especially around neurons (Marinesco, 1909)

Spleen The spleen may show hypertrophic and hemorrhagic or hyperemic follicles, with some central necrosis (Babès and Jonnesco, *1909 b*)

Pancreas The pancreas may present some epithelial desquamation of the excretory canals, and a pericanalicular infiltration (Babès and Jonnesco, *1909 a*)

neurofibrillary hypertrophy may be observed, particularly in cells of the spinal ganglia; the neurofibrils become hypertrophied, coalescent, and simplified.

Many degenerate nerve cells throughout the central nervous system, especially in the cord and spinal ganglia, are surrounded by leukocytes (see below, Babès nodules and Van Gehuchten and Nelis lesions), when the cell is completely destroyed neuronophagia occurs Negri bodies may be found in some of the less damaged cells (*vide infra*).

Inflammatory lesions. There is usually a well-marked leukocytic infiltration, diffuse and perivascular, in the spinal ganglia, cord (especially the gray matter), bulb and protuberances, and cerebellar and cerebral cortices. The infiltrating cells consist of lymphocytes, with a few plasma cells and polymorphs Thrombosis may occur in the vessels in the affected areas. Neuroglial reaction is usually present, especially in the region of the substantia nigra The astrocytes are mainly involved although the oligodendroglia react also, more particularly in the cord.

Babès nodules. These characteristic lesions of rabies, first described by Babès (see 1892 and 1912 *a*), have been demonstrated repeatedly by later workers (e g, Van Gehuchten and Nelis, 1900 *a*, *b*, Marinesco, 1909, Marie and Chatelin, 1919, Lowenberg, 1928, Bassoe and Grinker, 1930; Schukru-Aksel, 1934, 1935–6).

The lesions occur mainly in the cord and bulb where nerve cells are found to be surrounded by mononuclear phagocytic cells In the severest lesions neuronophagia takes place. The lesions are not in any way specific for rabies, of course, being merely the usual reaction of the leukocytic cells and microglia to degenerate neurons.

Van Gehuchten and Nelis lesions. Changes in the spinal ganglia were probably first noted in the latter half of last century. Thus, Nepveu (1872) recorded changes in the spinal ganglia of the affected side, he found that the ganglion cells were often surrounded and separated by leukocytes. He suggested that some of these leukocytes were probably epithelioid cells from the capsule of the ganglion cells. Similar changes were fully described and elaborated by Van Gehuchten and Nelis in 1900 (*a*, *b*), and the lesions are now usually known by their names Other workers have confirmed the presence of these lesions (e g, França, 1901, Marinesco, 1909, 1910, Marie and Chatelin, 1919, Dawydowski and Dwijkoff, 1928, Manouélian, 1936 *a*) The lesions consist of a collection of round cell leukocytes around a more or [illegible] ronophagia in the severer grades of the [illegible] advanced stage of degeneration it ma[illegible] Van Gehuchten lesion, the neurofibrillary network of the cell (Manouélian, 1936 *a*) The lesions are thus seen to be similar in nature to Babès nodules. Herzog (1945) has drawn attention to the presence of lesions in the ganglion nodosum of the vagus.

Negri bodies Numerous authors have recorded the presence of these characteristic inclusion bodies in man The bodies are eosinophilic structures measuring up to about 10 μ They may show an internal structure with granules of chromatin-like material. They are usually found in more or less undamaged nerve cells in the brain, particularly in Ammon's horn (hippocampus) They occur also in other parts of the central nervous system including the cord They have been found in the suprarenal gland. These bodies are fully described in a special section (see Ch LXXVI)

Having now studied the various individual changes that may occur, it remains to discuss their distribution in the central nervous system.

Meninges A slight degree of pial leukocytic infiltration, mainly perivascular, may occur, but quite often the meninges are normal. In other cases hyperemia may be present Lowenberg (1928) reported a case of an acute meningitis, definitely rabic in nature (see also Durand, 1941)

The cord The naked-eye lesions are usually those of congestion and some softening Microscopically a variety of lesions occurs Thus there is an infiltration

only 1 contracted the disease (McKendrick, 1940) showing that man to man transfer is very rare.

Animals which may transmit rabies to man.

Dogs. The most common reservoir of rabic infection throughout the world is in canines. The virus is spread amongst them by the bites of rabid members of the community. The canine virus has maintained the properties of street virus for probably over 2,000 years, although individual strains may differ slightly from each other in their characters. Canine rabies may sometimes exist in a locality without apparently giving rise to human cases (see, e.g., Bouffard, 1912, Blanchard and Lefrou, 1912).

In parts of Africa canine rabies is found, and this is occasionally transmissible to man. This infection was probably imported from Europe or elsewhere at some period and is quite distinct from another indigenous rabies, *oulou fato* or "mad dog" disease (see Heckenroth, 1918, Remlinger and Curasson, 1924, Hudson, 1944). *Oulou fato* is of definitely rabic nature and the virus has been quite thoroughly studied (see p. 812), it occurs in French West Africa, Senegal, and the Sudan. The affected dogs become paralyzed, and tend to segregate themselves, thus there is little opportunity for biting man. Human infection may, however, arise, and Nicolau, Mathis, and Constantinesco (1933) have isolated a strain.

The incubation period in dogs naturally infected is from 3–7 weeks, but may extend to somewhat over 6 months. The animal alters in disposition and shortly develops furious rabies (see p. 819). Pariah dogs are more likely to bite human beings than are domestic dogs. The dog is only infective if rabies virus is being excreted in the saliva, and it appears that in only about 50 per cent. of cases can virus be demonstrated in the submaxillary glands (Webster, 1942). Virus reaches the glands by passage along nerve trunks. The animal is probably infective for a day or so before clinical symptoms appear.

Cats. These animals are occasionally responsible for human cases of rabies (see, e.g., Remlinger and Bailly, 1930*c*, Wright and Gosden, 1946).

Wolves. Wolves are mainly concerned with the dissemination of rabies in Russia, Balkan, and Mid-European countries, and to some extent in France. The bites are usually very severe and energetic antirabies treatment is required.

Coyotes. Coyotes are responsible for the spread of rabies in California (Ledfors and Seiler, 1938).

Jackals. Jackals are common disseminators of rabies in India, as Kasauli statistics show, the bites usually being serious. They are responsible for about 30 per cent. of cases of rabies in India (Webster, 1944). They may also cause rabies in Northern Rhodesia (see Du Toit, 1936).

Skunks. In North America rabies may be contracted from the bite of a rabid skunk.

Viverridae. Animals of the mongoose family are being increasingly incriminated as transmitters of rabies. In India a number of bites has been inflicted by rabid mongooses. Thus at Kasauli from 1922 to 1933 there were over 30 such cases (see Grewal, 1932, 1933, Abbasi, 1943).

This family is probably of main importance, however, in South Africa, where the following members of the *Viverridae* may transmit human rabies. *Cynictis penicillata* (yellow mongoose), *Geneta felina* (genet cat), *Felis ocreata* and *nigripes*, *Suricata suricatta* (suricate), *Myonax pulverulentus* (pepper and salt meercat), and *Geosciurus capensis*. Interesting papers have appeared on this subject by South African writers (Thomas and Neitz, 1935–6, Du Toit, 1936, *Report*, 1936, Snyman and Thomas, 1938, Snyman, 1940).

Badgers and foxes. Rabies may follow the bite of rabid badgers and foxes (see, e.g., Lentze, 1931, Thomas and Neitz, 1935–6). There has been a number of out-

FIRST INTERNATIONAL CONFERENCE ON RABIES

A conference on rabies was held in Paris in April 1927, at *L'Institut Pasteur*, and was attended by the majority of leading rabies experts from all parts of the world, representing 26 countries Papers were read to the conference, and questionnaires previously sent out to antirabies institutes were considered. The official reports of the conference were compiled by MM. Marie, Remlinger, and Vallée. These have been published independently by the League of Nations (Marie, 1927 *b*, Remlinger, 1927 *b*, *c*; Vallée, 1927) and as a special supplement to the *Annales de l'Institut Pasteur* for 1927.

The conference adopted a number of resolutions which should be carefully studied, representing as they did the considered opinion of the world's leading authorities on the disease at that time.

EPIDEMIOLOGY

Geographical Distribution

There is no part of the world in which rabies may not occur. Thus, it exists in the northern regions such as Greenland and Iceland, temperate regions such as France, Germany, and other European countries, the United States and Canada, subtropical regions such as the Mediterranean littoral, tropical lands such as India, and the Far East.

With regard to Great Britain, in 1884 the incidence of rabies increased in London and the home counties, and in 1885 there were some 27 human deaths. Muzzling and surveillance of dogs were instituted with the result that by 1887 there were virtually no cases Great interest was taken in an outbreak of rabies among the deer in Richmond Park in 1886–7 (see Editorial, *Nature*, 1887–8), also one in Suffolk (Adami, 1889). From the Richmond Park outbreak the virus was isolated, on rabbit injection, by Cope and Horsley (1888).

After 1891 the muzzling laws were slackened or given little attention, and by 1895 there were again numerous cases of the disease. Reinforcement of the laws lessened the number of cases again, and from 1901 to 1918 there were no further cases in England After World War I came another outbreak, but muzzling soon brought this to an end, and since that time the disease has been virtually unknown In fact, since 1898 there have been only 2 deaths from rabies in England and Wales (*Memorandum*, 1944)

In America human cases occur sporadically, and a comparatively small number of deaths (under 100) occurs year by year Canine rabies is very common in some states (see Blatt *et al*, 1938, Denison and Dowling, 1939, Webster, 1942, *Publ Hlth. Rep*., 1947) On the European continent the disease is common, as it is in Algeria, Morocco, Tunisia, and other parts of North Africa In parts of Africa an indigenous form of rabies affects dogs (*oulou fato*) which is rarely transmissible to man (see pp 801 and 812 *Oulou fato* is not the common type of rabies of French West Africa, where strains of virus are usually of full virulence (Lépine *et al*, 1939) In South Africa the disease is not prevalent, but a few cases occur from time to time, usually following the bites of animals of the mongoose family (meercats, etc, see p. 801) The disease is very prevalent in India, Indo-China, and the Far East

Method of Spread of Rabies

The vast majority of cases occurs following the bite of a rabid dog or other animal, and a full list of animals which have transmitted rabies to man will shortly be given. In certain cases, however, the animal may not actually bite, but only scratch or claw the skin In all such cases rabies may follow, though only comparatively rarely Cases have occurred where a child has bitten a parent, necessitating anti-rabies treatment (see Pampoukis, 1900), of 8,581 persons in contact with rabies

are discussed on p 830.) Following local treatment, a course of antirabies treatment should be instituted forthwith (see Ch LXXVIII).

General Measures for the Control of Rabies

The control of rabies on a large scale is a complicated matter and involves attention to a considerable number of different points. Each country has varying rules and regulations and there is, as yet, no international agreement as to the best method of limiting the spread of the disease The procedure followed in Great Britain during the 1918 outbreak has been described (*Memorandum*, 1944) The reader is also referred to the valuable official publication of the Government of India (Webster, 1944), and the paper of Tierkel (1948) on the plans of the United States Public Health Service.

(*A*) *Destruction of animals* A number of countries enforce destruction of all rabid animals and those animals bitten by them. The following regulations, as used in Palestine (Stuart and Krikorian, 1925), are given as a model

1 Every rabid animal to be destroyed,
2 Animals bitten by rabid animals to be dealt with as follows
   (*a*) monkeys, dogs, cats, to be destroyed,
   (*b*) camels, bulls, cows, calves, sheep, goats to be slaughtered, the carcasses may be sold for food,
   (*c*) valuable animals to be destroyed, isolated, or given antirabies treatment
3 Every animal bitten by a suspectedly rabid animal to be destroyed or isolated Dogs in contact with other dogs, suspectedly rabid, should be destroyed or isolated
4 All animals that have bitten a human being are to be isolated and closely watched for at least 10 days, from the point of view of treatment, it is most important to know whether the person has been bitten by a genuinely rabid animal or not
5 Stray dogs and jackals should be destroyed

(*B*) *Muzzling*. Muzzling always markedly reduces the number of cases of rabies spread by infected dogs (for an illustrative chart, see Harvey and McKendrick, 1930). The order must be enforced strictly and backed up by measures to deal with stray dogs In New York, leashing has been allowed as an alternative to the more irksome muzzling (see Olesen, 1935).

(*C*) *Quarantine* The practice in many island countries is to quarantine for some months all dogs entering the island This measure is usually remarkably effective An interesting example of what may happen when rabies is introduced into a small island previously free was afforded in the case of Madeira in 1894 (Goldschmidt, 1894) The disease spread rapidly among dogs, goats, and cats, and there were some human cases It was suspected that some dog imported to the island must have been incubating rabies on arrival There have been similar outbreaks recently, for instance in Corsica, traced to the entry of soldiers' pet dogs (Lépine, 1946)

In Great Britain at the present day, strict quarantine is imposed on dogs imported into the country, 6 months observation being enforced Galloway (1945) reports that in 21 years 16 cases of rabies have developed in quarantined dogs, and the 6 month period is fully justified

(*D*) *Education of the public* In any area where rabies is endemic an effort should be made to teach the public the exact epidemiology of the disease They should be encouraged not to keep dogs, but if they must, muzzling or leashing should be insisted on The public should be trained to report all dog bites to the health authority In this way the person bitten can be subjected to antirabies treatment at once, and the offending animal removed for competent veterinary exami-

breaks of rabies in foxes in the southern states of the United States (Sellers, 1941; Johnson, 1945; Public Health Reports, 1947).

In New York State in 1946 there were 308 reported cases in foxes, and 440 in cows, the fox rather than the dog appears to be the central figure in rabies epidemiology, with cattle being infected by the bites of foxes (Korns and Zeissig, 1948).

Mice. Remlinger (1905 *c*) recorded a case following a mouse bite.

Birds. Rabid birds may (rarely) attack human beings, necessitating antirabies treatment (see Kraus, Gerlach, and Schweinburg, 1926, Remlinger and Bailly, 1929 *a*).

Other animals Harvey and McKendrick (1923) give the following list of animals which have been known to bite when rabid. horse, ox, buffalo, hyena, panther, camel, goat, deer, elephant, bear. The rabid ass and calf may also bite, but it must be emphasized, from the practical point of view, that ruminants and solipeds are practically never concerned in the causation of human rabies. For example, of 26,802 persons bitten by solipeds and ruminants, and subjected to antirabies treatment, only 3 developed rabies (McKendrick, 1940) Smith *et al* (1938) reported that for the past 30 years no death from hydrophobia was recorded from the Kasauli Institute and its out-stations, from bites of horses, cows, donkeys, or buffaloes

Denison and Leach (1940) examined 500 rats in Alabama, but failed to isolate virus by mouse inoculation.

The infectivity of rabid animals.

In the great majority of cases the infection is transmitted by means of the saliva of the biting animal. It has often been demonstrated that the saliva is infective during the period of the declared disease (see p. 826) Further, a number of observations has shown that the saliva of dogs may be infective several days before the appearance of symptoms of rabies (see p 827). Therefore, in countries where rabies occurs, any dog bite should be regarded as possibly that of an infective animal

Rabies developing apart from a bite must be considered as exceptional, although there have been a few such observations (see above).

According to some observations in America (see Webster, 1937), it is probable that dogs may carry the virus in the central nervous system without showing typical symptoms of rabies Thus the virus has been isolated (by mouse inoculation) from dogs showing fits and other indefinite, not characteristically rabic, symptoms. There is no evidence, however, that dogs can act as symptomless carriers (Johnson, 1943)

Personal Prophylaxis

The wound should be encouraged to bleed According to Mackie (1928) it is advantageous to wash out the wound with acriflavine, and then pack with gauze soaked in the antiseptic Prompt cauterization with a thermocautery, fuming nitric acid, or phenol is usually recommended (Ekstrom, 1830), but there is little evidence that such drastic agents are more effective than milder antiseptics such as iodine or even simple soap washing (Shaughnessy and Zichis, 1943) The general opinion is that cauterization has a definite value, but our attempts to obtain recent accurate figures to support this statement from various Pasteur Institutes have proved fruitless Some years ago Harvey and McKendrick (1923) found that efficient cauterization lowered the mortality rate in the ratio of 4 3 Experimentally it has been shown that cauterization of infected animal wounds, although usually effective up to half an hour after the infliction of the injury, may fail, even within 5 minutes (Babès and Talasescu, 1894) (The effects of cauterization on experimental rabies

of brain may be removed and pressed firmly down onto the slide by a coverslip. (Instead of removing the bone with bone cutters, it is safer to smash it with a heavy blow, before the skin is reflected. Pieces of bone may then be picked off, and the brain exposed.)

Microscopical examination.

1. *Wet films.* The debris on the lancet used to scrape the hippocampus is floated out in dilute acetic acid. The preparation is then examined wet and unstained for the characteristic inclusions (see Negri-Luzzani, 1913).

2. *Stained films.* Scrapings from the hippocampus may be stained by Giemsa's stain as a film preparation. Webster (1944) prefers a modified Van Gieson stain as follows: the gray matter is crushed and spread on a slide by pressure under a coverslip. Without drying, the film is fixed in methyl alcohol for 2 minutes. Then stain for 5 minutes with the following stain freshly mixed. (*a*) 0.3 c.c. saturated alcoholic basic fuchsin; (*b*) 2.0 c.c. saturated alcoholic methylene blue, (*c*) 30 c.c. aq. dest. Then wash rapidly in water, and dry. In such films the Negri bodies are generally found lying free. Sellers' method is popular in America (1927).

3. *Preparation of sections.* Small pieces of the horn are fixed for 2 to 4 hours in formol saline, spirit, or Zenker's solution. The tissue is then taken to paraffin in the usual way as rapidly as possible. Sections are cut so that the face is a frontal section of the whole horn, they should be stained by Mann, Hamilton, Lépine or
[illegible] The specimen
[illegible] formol saline,
[illegible] er cent., 90 per
cent., and absolute alcohols, acetone, xylol, 2 changes of paraffin. After blocking and sectioning, Van Gieson's stain (as above) is applied for 3 minutes with the usual technique applicable to paraffin sections.

In addition to the search for Negri bodies in Ammon's horn, it is well to have sections made from various other parts of the central nervous system. These should be subjected to a general histological examination as well as searched for Negri bodies. Babès lesions are present in a [illegible]
occur in the bulb or cord which a [illegible]
1908, Babès and Stefanesco, 1908). [illegible]
ganglionic changes, especially in dc [illegible]
infected with rabies (França, 1900) [illegible]
amining the plexiform (inferior v: [illegible]
lesions (see also Manouélian, 1942).

Biological examination.

Portions of the bulb and other basal parts of the brain are ground up in sterile saline and lightly centrifuged. The supernatant fluid is injected subdurally in rabbits, guinea-pigs and mice. Although the distribution of virus may be somewhat irregular, it is rarely a[illegible]
and Bailly, 1931 *d*). T[illegible]
decomposed specimer[illegible]
1939–1940) or Seitz-fi[illegible]

[illegible] Microscopically, well-developed Negri bodies can be found. The test is best performed by injecting 6 mice intracerebrally. Three are killed on the 5th, 6th and 7th days respectively and the brains

nation. As found necessary, it may be destroyed forthwith, isolated, or returned to its owner to be reexamined later (see, e.g., Olesen, 1935).

(*E*) *Prophylactic vaccination of animals* This subject is discussed in Ch. LXXVII.

(*F*) An interesting account of the very varied measures to control rabies followed in most of the states of America and the provinces of Canada will be found in *Public Health Reports*, 1947, *62*, 1215. In brief, the measures recommended include. (1) The introduction of a national plan for rabies eradication (2) Regulation of interstate transport of susceptible animals (3) Regulations governing the importation of susceptible animals from abroad to be administered by the Public Health Service. (4) All cases of rabies, human and animal, to be reported. (5) Annual licensing of dogs to be introduced. (6) In urban areas, the granting of a license to be made contingent on vaccination. (7) Vaccination of dogs by annual injection (see p. 848) (8) The establishment of quarantine during outbreaks of rabies in dogs, proper disposition of rabid and suspectedly rabid dogs, destruction or supervised detention for not less than 3 months of dogs known to have been bitten by or exposed to rabid animals, and the impounding and disposal of all stray dogs.

LABORATORY DIAGNOSIS

Human Rabies

The laboratory examination of persons dead as a result of suspected rabies involves 2 lines of approach First, the hippocampus should be dissected out, films made and suitably stained (*vide infra*), and the remainder fixed prior to histological examination for Negri bodies (see Ch LXXVI). Other portions of the central nervous system also should be examined histologically. Secondly, a suspension of the bulb should be made and injected intracerebrally or subdurally in rabbits It is as well to prepare suspensions from several areas of the central nervous system, as the distribution of virus may be patchy (Koch, 1929) If a virus agent is isolated, it should be tested for its effect by other routes of injection, and on other animals Mice may also be injected and Negri bodies searched for in their brains (Webster, 1936, 1937, *vide infra*). Tissue cultures may be inoculated (Plotz and Reagan, 1942) It is always worth while considering the possibility of rabies in any obscure nervous case (Schaeffer and Leider, 1941–2).

Canine Rabies *

When a person has been bitten by a dog or other animal, steps should always be taken to discover if the animal is actually rabid or not If living, it should be isolated to see if rabies develops, if dead, the head of the dog should be sent to the laboratory packed in ice, or failing this in glycerol or salt If neither of these substances is available, it should be remembered that Negri bodies resist putrefaction for some time Various methods are in use for the examination of the head, but they mostly resemble the method of Negri-Luzzani (1913)

The skin is slit in the middle line from the occiput forwards to the muzzle, and the skin flaps are reflected to the level of the ears The exposed skull bone is then removed with a suitable cutting instrument The dura is cut and the cerebral hemispheres exposed The brain is removed (or it may be left *in situ*) and the superficial parts of the hemispheres sliced away horizontally, down to the level of the corpus callosum A dissection is then made into the temporal portion of the lateral ventricle to expose Ammon's horn (hippocampus) The horn is dissected out and cut perpendicularly with a razor, then a lancet is scraped fairly deeply over the cut surface and the scrapings examined microscopically Alternatively, a small piece

* In Great Britain specimens may be sent to the Veterinary Laboratory of the Ministry of Agriculture and Fisheries, Weybridge

CHAPTER LXXIV

TRINIDAD RABIES

Trinidad rabies was first brought to general notice by an official report (*Report*, 1931)[1] and by a paper of Hurst and Pawan (1931). The latter reported 17 cases (all fatal) of acute ascending myelitis The patients came from Siparia in S.W. Trinidad, were natives, and mostly school children. Infection by the bite of vampire bats was suspected, as almost all cases had a definite history of having been bitten on the feet or lower limbs. Pawan (1936 *a*) reported that at that time there had been over 50 human cases, all of whom died. Later (1939) he reported the occurrence of 73 cases to date.

CLINICAL FEATURES

The clinical features have been described from time to time (Hurst and Pawan, 1931, 1932; De Verteuil and Urich, 1935–6, Pawan, 1936 *a*).

Incubation period. Symptoms usually develop from 3 to 4 weeks after the bite of the bat

Onset The onset is characteristically sudden The patient is febrile and often has a headache The bitten foot or leg usually feels burning and tingling, and may actually be paresed at the onset of the disease.

Course. After these prodromal symptoms, which may last for 1 to 4 days, the muscular weakness progresses to complete paralysis of the leg with anesthesia and loss of reflexes. The trunk muscles are soon involved also, and the paralysis spreads to involve the arms and finally the bulbar nerves. The temperature may be raised Excessive salivation usually occurs, but only in the terminal stages are there hydrophobic symptoms

PATHOLOGY

The pathological features have been described in detail by Hurst and Pawan (1931, 1932). Naked-eye, the cord appears congested and soft, especially in the cervical and dorsal regions. The brain also is congested The heart may show [illegible] re is [illegible] s of [illegible] de- [illegible] ears [illegible] tion of the sheath

EPIDEMIOLOGY

The disease tends to occur in small localized epidemics which last from 1 to 10 weeks, the number of cases in any one epidemic has varied from 13 to 43 (De Verteuil and Urich, 1935–6). As regards seasonal incidence, 64 per cent. of cases have occurred from May to August, in the rainy season (De Verteuil and Urich). Investigations have conclusively proved the disease to be spread by the bat (see, e.g., Hurst and Pawan, 1931, 1932; Metivier, 1935; De Verteuil and Urich, 1935–6, Pawan, 1936 *a*, *b*, 1937).

[1] References are appended at the conclusion of Ch LXXIX, p. 857 et seq

examined for Negri bodies. The remaining 3 animals are kept for a month to see if rabies develops. Animals may also be injected in the tail (Jonnesco, 1934 *a*).

Tissue cultures also may be inoculated

To obtain a positive diagnosis in the largest possible number of cases, it is essential to examine the canine material for Negri bodies as well as attempting to isolate virus by animal inoculation. A significant number of specimens in which Negri bodies cannot be found will prove positive on animal inoculation, and to a lesser extent some specimens negative by animal inoculation contain Negri bodies (Damon and Sellers, 1941–2, 1942).

In the cord, changes occurred in the lumbar portion, where nerve cell degener-

in the lumbosacral portion

The lumbar ganglia showed the more severe inflammatory infiltration on the inoculated side, but the nerve cell degeneration was equal on the 2 sides

Negri bodies were found regularly. They measured from 2 μ to 15 μ or rarely 25 μ, and occurred more profusely in Ammon's horn than elsewhere.

Hurst and Pawan made a comparison of these histological findings with those produced by *virus fixe* Following intracerebral inoculation with *virus fixe* the changes were more distinctly inflammatory. The lesions of *virus fixe* were as severe in the basal nuclei as in the cortex, this did not obtain with Trinidad virus, where the basal nuclei showed less change than the cortex. The changes in the cerebellum also were severer with *virus fixe*.

Rabbits Rabbits can also be infected with Trinidad virus, by intracerebral injection (Hurst and Pawan, 1931, 1932, Andrews, 1933) The symptoms are those of paralytic rabies, although Remlinger and Bailly (1932 *a*) noted a fairly prolonged stage of motor excitement prior to paralysis Histologically the cerebrum showed meningeal cellular infiltration, perivascular cuffing, and microgliosis (Hurst and Pawan, 1932) The nerve cells showed all stages of degeneration The brain stem showed m[illegible]

bellum sho[illegible]

tion, and n[illegible]

Pawan [illegible]

Rabbits w[illegible]

bodies were not found.

Guinea-pigs. Andrews (1933) has reported that these animals are more susceptible to Trinidad virus than to ordinary rabies virus Apparently after injection they may develop the furious form of rabies (Remlinger and Bailly, 1932 *a*)

Dogs After intracerebral injection the animals develop paralysis of the posterior limbs, and some may bite (Remlinger and Bailly, 1932 *a*)

Other animals. Remlinger and Bailly (1932 *a*) reported that the virus could be transmitted to the following animals cat, buzzard, rat, mouse, *Meriones shawi*, gerbille, and hen

Antigenic Structure

Rabbits immunized by killed *virus fixe* were protected against a subsequent injection of Trinidad virus On the other hand, Trinidad virus did not confer much resistance against a subsequent injection of *virus fixe* (Hurst and Pawan, 1932; Pawan, 1936 *a*)

Trinidad Rabies in Cattle

In 1925, cattle near Port-of-Spain were found to be affected by an unusual disease, dying of a form of bulbar paralysis with ataxia, salivation, and constipation. By 1926 it had spread farther west to Diego Martin, and by 1935 all parts of the colony were infected (Metivier, 1935) By 1936 about 2,000 animals had died from the disease (Pawan, 1936 *a*)

The animals most commonly involved, apart from cattle, are horses, mules, donkeys, goats, sheep, and pigs By 1930 to 1931 the disease had assumed a somewhat *different* clinical form from its original, now it is more of an acute ascending myelitis than a bulbar paralysis The animals salivate, stop grazing, and are ataxic, by the 3rd day of the illness they lie down and usually die within a day or two.

It was realized that the disease closely resembled the paralytic rabies of bovines in

The Rôle of the Bat

Species of bat incriminated. The main vector of Trinidad rabies is *Desmodus rotundus murinus* (Wagner, 1840) This bat is a blood-lapper, and its habits have been described by various writers (in particular by De Verteuil and Urich, 1935–6). The animal measures about 13 inches from wing to wing, and has very sharp teeth with which it pierces the skin; it prefers to bite dark skin to pale. The blood oozes out and is caught in the bat's mouth The sole food of the desmodus bat is blood, bovine or human, its normal food is bovine blood, and it only attacks men if animals are shut away. It has often been noticed that stabling of live stock results in an outbreak of human cases. The bat bites only in the dark, while during the day it retires to sleeping places in caves, trees, and under bridges It deposits a characteristic tarry excrement

Another species of bat—the fruit-eating bat, *Artibeus planirostris trinitatis*—has been found to carry the virus of rabies. Although this animal does not attack man it may act as a reservoir of infection for a community of desmodus bats (De Verteuil and Urich, 1935–6)

Natural rabies in bats. Various Trinidad observers have noted that desmodus bats may develop clinical manifestations of furious rabies, and frequently fight with each other. The saliva and salivary glands have been found to be infective (Pawan, 1936 *a*), and there is no question that the disease is spread from bat to bat, as well as to man or animals, by bite

Probably bats may harbor the virus without showing any obvious manifestations of infection (Pawan, 1936 *b*).

Fruit-eating (artibeus) bats may fight with desmodus bats and transmit the infection to them, but artibeus bats do not attack man

Infectivity of the bat Numerous observations have been carried out on bats caught in Trinidad by Pawan (see 1936 *a*). The virus of rabies has been isolated, by inoculation of monkeys and calves, from the brains of both desmodus and artibeus bats infected under natural conditions. Also, Negri bodies have been found in approximately 4 per cent. of bats—mainly desmodus, but sometimes artibeus.

Preventive measures involve burning bright lights and erecting nets to keep away the bats, but these procedures are not very effective. More hope is to be expected from the intensive campaign of bat destruction that is being practiced on the island

PROPERTIES OF TRINIDAD RABIES VIRUS

Animal Inoculation Experiments

Bats. Bats can be inoculated experimentally (intracerebrally) and may develop furious rabies, paralytic rabies, or may harbor the virus without developing symptoms (Pawan, 1936 *b*).

Monkeys The disease may be transmitted to monkeys by intracerebral injection (Hurst and Pawan, 1931, 1932) After 7 to 9 days the animals become aggressive, have violent muscular spasms or convulsions, and die within about 12 hours Intrasciatic injection also secures infection

The histological features have been described *in extenso* by Hurst and Pawan (1932) Thus, in group 1 the inflammatory changes were only slight. Microglial foci, nerve cell degeneration, and perivascular infiltration might all be found in the cord and higher parts of the central nervous system The spinal, as well as gasserian and sympathetic, ganglia showed marked changes Many nerve cells were degenerate or actually necrotic, and often replaced by capsular cells, there was also a diffuse cellular infiltration. In group 2 inflammation was more marked The cerebrum showed some meningeal infiltration and perivascular cuffing Microglial foci were well developed, consisting of proliferated microglial cells and a few lymphocytes At the periphery infiltration was more diffuse

CHAPTER LXXV

THE RABIES VIRUS

STRAINS OF RABIES VIRUS

A. Street Virus

THE strain of rabies virus found in nature, in man, and animals, is known as street virus, or *virus de rage de rue*. When street virus is passaged through animals by intracranial injection, it eventually becomes modified in its properties, and is then known as fixed virus or *virus fixe*.

Strains of street virus vary greatly in the rapidity with which fixation occurs, as represented by the standardization of the incubation period and the increasing rarity with which Negri bodies develop

Stuart and Krikorian (1931)[1] have suggested that street viruses may be classified into various groups.

1. These strains show short incubation periods *de novo* and are true *virus de rue renforcé* strains
2. (*a*) Strains of high virulence in which complete fixation occurs after a few passages.
   (*b*) Ordinary strains of street virus in which fixation occurs in about 50 passages
3. Strains of low virulence where fixation does not occur even after prolonged passage.

1. *Virus de rue renforcé.*

This term is applied to certain strains of street virus of unusual virulence isolated from various sources (Calabrese, 1896, Remlinger, 1926 *b*, Harvey and Acton, 1922–3, Alivisatos, 1926, Calderini, 1928, Téodorasco, 1928, Koch, 1929, Jonesco, 1930 *a*, Stuart and Krikorian, 1931, and others, *vide infra*).

General characters

Incubation period in man It has been suggested that the shortness of the incubation period in various human cases is due to infection with one of these *renforcé* strains (see, e g, Alivisatos, 1926, Bablet and Joyeux, 1930, Genevray and Dodero, 1934) Proca and Bobes (1940), however, on the basis of an extensive experience concluded that there was no parallel between the incubation period in untreated persons and the incubation period in rabbits inoculated intracerebrally with brain suspensions of these patients

Animal inoculation. In rabbits, after subdural inoculation, the incubation period is much shorter than the usual 15 or more days characteristic of street virus. Symptoms of paralytic rabies usually develop within 10 days of infection, and possibly even after about 3 to 5 days. These strains may prove of normal virulence for the guinea-pig (Remlinger, 1925 *b*) Negri bodies are usually completely absent. Fixation occurs in rabbits after very few passages, perhaps only one or two (Jonnesco, 1938 *b*).

Properties of individual strains.

A large number of strains falling into group 1 (Stuart and Krikorian) has been isolated, and the following are the best known

(*a*) *Tangier B strain*, (*b*) *Puymyrol*; (*c*) *Soucy* (see Stuart and Krikorian).

[1] References are appended at the conclusion of Ch LXXIX, p 887 et seq

South America, and it was soon found that it was spread by the vampire bat *Desmodus rotundus* As the coast of South America is only 10 miles from Trinidad, it is possible that in the first place infected bats flew or were carried by ship.

Negri bodies can be found in the central nervous system of the paralyzed animals, and rabies virus can be obtained from the central nervous system or saliva by injection of monkeys, rabbits, or other susceptible animals (Hurst and Pawan, 1932; Pawan, 1937) These facts definitely establish the rabic nature of the infection.

(Q

The cerebrum showed some meningeal round cell infiltration, and perivascular cuffing was noted in the gray matter, and to a lesser degree in the subcortical white matter. Microglial proliferation occurred in the gray matter. There was some satellitosis, but no neuronophagia.

The cerebellum showed some degeneration of Purkinje cells, microgliosis, and the presence of Negri bodies. The pons showed much perivascular cuffing, microgliosis, and nerve cell degeneration. The cord showed rather patchy changes, but microgliosis, perivascular cuffing, nerve cell degeneration, and Negri bodies were all found.

South American Bovine Paralytic Rabies (mal de caderas)

For some years a paralytic disease of horses and cows, which is known locally as *mal de caderas,* has been prevalent in South America. Numerous papers have appeared on the subject (see, e g, Carini, 1911; Haupt and Rehaag, 1921, Rosenbusch, 1930, Remlinger and Bailly, 1931 *a*, Migone and Peña, 1932, Quiroga *et al*, 1932; Pawan, 1936 *a*, Molina, 1938, Everling, 1939) Those who are interested will find fuller references in the *Tropical Diseases Bulletin* and in the *Boletín* for 1936

The disease enjoys a wide distribution over the continent of South America, occurring in Santa Catharina (S Brazil), North Brazil, Paraguay, Argentine, British Guiana, Venezuela, Bolivia, and probably elsewhere

Horses and cows are most commonly affected and develop an ascending paralysis resulting in paraplegia The fatality rate is from 20 to 60 per cent. The disease is usually contracted at night, and animals in stables generally escape The infection is transmitted by blood-lapping (desmodus) bats, and the virus of rabies has been isolated from naturally infected bats

The rabic nature of the disease was first established by Haupt and Rehaag in 1921 Negri bodies occur in the brains of the affected bovines, and rabies virus can be isolated also by suitable animal inoculation The infection can be transmitted experimentally to bovines by injection of virus

In laboratory animals the symptoms are those of *virus fixe* Thus, Remlinger and Bailly (1931 *a*) found that rabbits, guinea-pigs, rats, mice, dogs, sheep, hedgehogs, jackals, and buzzards were all susceptible, and developed paralytic rabies

Mexican Derriengue

Johnson (1948) has described the main features of this fatal paralytic illness of cattle on the Pacific coast of Mexico, that may occur in the form of epizootics Rabies virus has been isolated from a cow and from vampire bats

authors (Remlinger and Curasson, 1924; Remlinger, 1926 *b*, Jadin, 1939). Further, a human st

Fixation

eventually

as passage

infectivity (as tested by dilutions) is increased approximately 40 times by fixation, *virus fixe* resists glycerol for 3 to 4 months whereas street virus only resists for 3 to 4 weeks.

By passage of a street strain of *oulou fato* through rabbits 2 different fixed strains were isolated, one having an incubation period of 8 to 10 days, the other 10 to 12 days (Nicolau, Mathis, and Constantinesco, 1932).

Animal inoculation. Rabbits injected subdurally with street virus die after about 18 days from paralytic rabies, often with a preliminary phase of excitation. Fixed virus produces a purely paralytic disease. Animals can also be infected with street virus by the dermal, muscular, corneal, sciatic routes, and by the posterior chamber of eye.

Guinea-pigs also die of paralytic rabies, from infection with both street or fixed viruses Monkeys, rats, mice, dogs, and hens can all be infected with *oulou fato* virus.

Pathology. The pathological features of infected animals do not differ from those usually found in experimental rabies.

Cross immunity tests have established the complete identity of *oulou fato* virus with classical strains of rabies virus.

B. Miscellaneous Strains

herpetic (Luger

was soon proved

virus was found to be

The histological pictur

tests detected no relationship between Koritschoner's virus and Doerr's or Levaditi's herpes strains, these tests showed, moreover, that Koritschoner's virus was identical with rabies virus (Takaki, 1926)

2. *Kobayashi's (1925) virus.* This strain was isolated from a case of Japanese encephalitis in 1924. Kobayashi himself realized that the strain was very similar to rabies The proof was completed by Cowdry (1927) by animal and cross immunity tests.

3 *DK virus* This strain, thought to be herpetic, was shown to be rabic by cross community reactions (Nicolau and Kopciowska, 1929)

4 *Virus ECK* (*Silberstein*) This virus was isolated from the brains of dogs operated on to produce Eck's fistula and its precise nature seems to be in some dispute, some claiming it to be a rabic strain (see Remlinger and Bailly, 1930 *i*)

5 The virus known as Moscow 2 appears to be a rabic strain (Howitt, 1941).

C. *Virus Fixe*

The process of fixation.

Street virus is usually fixed by passage intracranially through rabbits. With the progress of time, the usual 15 to 21 day incubation period of street virus becomes shorter and shorter until it eventually reaches 6 to 8 days (see, e.g., Pasteur, Chamberland, and Roux, 1884, Hoegyes, 1888, Celli and Marino-Zuco, 1892, de Blasi and Travalli, 1894)

As has been mentioned above, strains of street virus vary greatly in the ease with which they become fixed, some becoming so almost at once, other strains

(*d*) *Chisinau strain.* Remlinger and Bailly (1930*a, b*) reported on the properties of this Roumanian strain. The incubation period in rabbits was 7 to 8 days after subdural inoculation, and virus was also infective intramuscularly, subcutaneously, and intra-ocularly. The following other animals were infected: the dog, guinea-pig, cat, jackal, fowl, pigeon, and buzzard. Cross immunity tests (with fresh or etherized virus antigens) showed that the Roumanian strain was identical antigenically with the street virus of Tangier and with *virus fixe.*

(*e*) *Safad strain.* This strain of *virus renforcé* was isolated in Palestine from a human case bitten by a jackal (Stuart and Krikorian, 1931).

(*f*) *Jonnesco's (1932) strain.* Jonnesco (1932 *c, d*) isolated a strain of *virus de rue renforcé* from a rabid wolf. Inoculated intracerebrally in rabbits the incubation period was only 3 days, and in guinea-pigs 2 days Dogs and cocks were the only animals in which Negri bodies could be found. The strain was shown, by cross immunity tests, to be identical with ordinary street and fixed viruses.

(*g*) *Jonnesco's (1938 a, b) strains.* The same worker isolated 4 more strains from persons who died of rabies despite antirabies treatment. These strains were highly pathogenic to mice.

(*h*) *Bablet and Marneffe's (1932) strain.* This strain was isolated from a person who died in Hanoi in less than 20 days, although subjected to antirabies treatment. A strain of virus, killing rabbits in less than 8 days, was isolated from the bulb.

(*i*) *Pawan's (1938) strain.* This strain was isolated from a rabbit bitten by a desmodus bat. Rabbits were killed in 2 days and no Negri bodies could be demonstrated (see Ch. LXXIV)

(*j*) *Proca and Bobes' strains.* These workers reported on 389 strains of street virus and found that 40 per cent. had incubation periods in rabbits inoculated cerebrally of under 15 days.

2 (a) Strains ***(of virus renforcé).***

(*a*) *Gréen strain,* (*b*) *Tétuan strain;* (*c*) *Virus Tangerois-Martin;* all isolated from rabid dogs (see Remlinger, 1926 *b*).

(*d*) *Strain CN* from Bucharest (Levaditi, Lépine, and Schoen, 1929).

(*e*) *Odessa strain* This strain was isolated by Palawandow and Serebrennaja (1933) from the saliva of a patient bitten by a dog. In guinea-pigs inoculated subdurally, the usual incubation period was about 2 to 3 days, although somewhat variable. Fixation occurred after 29 passages.

In these strains (*a–e*) fixation usually takes 15 to 30 passages, when the incubation period becomes stabilized and Negri bodies no longer develop Apart from rapidity of fixation, the characters resemble those of ordinary street virus 2 (*b*) strains

(*f*) In a somewhat different category from the above strains are those of Trinidad rabies and *mal de caderas.* These strains form Negri bodies readily but, on animal inoculation, possess other characteristics of *renforcé* strains (see Ch. LXXIV).

2 (b) Strains.

This group contains the bulk of street viruses, and is the group generally referred to when one talks of "street virus" without additional qualification. The properties of ordinary street virus have been described in great detail in the literature, and many resemble those of fixed virus These characteristics are described below, and form the major part of this chapter

3. Strains of low virulence.

We shall describe in this section the virus of *oulou fato,* a strain of low virulence which can, however, usually be fixed after numerous passages. These strains from indigenous mad-dog disease of North Africa have been investigated by certain

2. Negri bodies are found regularly in street virus infections but only under special circumstances with fixed virus (see Ch. LXXVI). Lentz bodies are only found with *virus fixe*.
3. The rate of multiplication of street virus in the central nervous system is said to be less rapid than is the case with *virus fixe*. Fixed virus usually appears to be the more infective when tested in serial dilutions, thus, certain strains of *virus fixe* have an infective titer of 1/500,000 to 1/900,000, while [illegible] 2,000 to 1/100,000.
4. [illegible] lent as the white, [illegible] virulent (Harvey and Acton, 1922–3).
5. Fixed virus has an attenuated affinity for tissues other than the central nervous system. Injection of fixed virus subcutaneously usually fails to infect dogs, although rabbits are more susceptible
6. Dogs infected with street virus develop furious symptoms, whereas with fixed virus, paralytic rabies is noted
7. Guinea-pigs infected with fixed virus usually develop paralytic rabies, but with street virus some other manifestation occurs (see p 822).
8. Fixed virus is of low virulence for man, although probably not completely harmless (see neuroparalytic accidents, Ch. LXXIX).
9. When *septinévrite* is induced by intraneural or intracerebral injection, fixed virus does not disseminate throughout the nerves nearly so regularly as does street virus.
10. Remlinger and Bailly (1943 *b*) have made the suggestion that street virus may be considered as a "rough" or less virulent variant of fixed or "smooth" virus.

Simultaneous infection with fixed and street viruses.

Marie (1930) injected rabbits intracerebrally with a mixture of street and fixed viruses, and found that half died with the characteristics of one infection and half with the other. Plantureux (1933), on the other hand, found that rabbits so injected usually died from fixed virus infection (see also Bozzelli, 1935).

Injected intra-ocularly, the mixture produced symptoms after 10 to 15 days, whereas *virus fixe* on its own was noninfective, and street virus did not cause symptoms for 18 to 27 days Guinea-pigs injected with mixtures of the two strains died from *virus fixe* infection (Marie, 1930).

Special strains of *virus fixe*.

Most of the properties of the ordinary strains of *virus fixe* are similar to those of street virus and are fully described below. In addition to the ordinary strains of *virus fixe*, however, some with unusual properties have been described, and these will first be mentioned.

1 *Sassari fixed virus.* This virus, maintained at the Sassari Anti-rabies Institute for many years, has been used in the manufacture of Fermi's vaccine. Its properties differ from the usual *virus fixe* strains in certain respects and have been studied in particular by Fermi (1907), Repetto (1908), and Lépine and Sautter (1936) Thus, Negri bodies are readily developed in rabbits on intracerebral injection, rats are regularly killed on subcutaneous injection, and mice are very susceptible to the virus even by ingestion.

2 *Remlinger and Bailly's* (1935 d) *strain.* These authors investigated a strain of *virus fixe* in use in an institute where there appeared to be an unusual number of neuroparalytic accidents occurring They found that it was unduly resistant to desiccation, even 17- to 18-day cords proving infective. Further, a 3-day cord remained virulent in glycerol for 44 days, and in ether for 280 hours.

3. *Brussels strain* Le Fèvre de Arric and Tchang Kouo-Ngen (1924) compared the properties of the Brussels and Paris strains of *virus fixe*, and found that the

after about 20 passages, the average being about 50 passages, and some strains may never become fixed at all. Fixation may also be carried out by passage through dogs, monkeys, guinea-pigs, or cats. An unusual occurrence was reported by Helman (1888), who attempted to fix a *strain of street virus by rabbit subdural* passage. Despite 60 passages no fixation developed. With the 11th-passage brain, however, he initiated another parallel series of passages. This "branching-off" proved successful and fixation occurred with this line of virus.

The stability of *virus fixe*.

A number of attempts has been made to convert *virus fixe* back to street virus, and certain of these have been partially successful. Generally speaking, however, the properties of *virus fixe* are remarkably stable and alterations in behavior are very rarely observed. For instance, although an earlier report suggested that intracranial passage through dogs might somewhat alter the properties of *virus fixe* (Nicolle and Burnet, 1924), this was later denied (Nicolle and Balozet, 1932).

Passage of *virus fixe* through hedgehogs results in an increased virulence for the rabbit, the production of furious rabies in the dog, and the capacity of once more forming Negri bodies Another line of approach has been to treat *virus fixe* with chemical agents in an attempt to change its nature. Thus it has been reported that, by suspending *virus fixe* in bicarbonate of soda, furious rabies develops in animals instead of the usual paralytic symptoms (Remlinger and Bailly, 1936 *c*). Further, Jonnesco (1939 *a*), after storing fixed virus in glycerol for 144 days, found that, after inoculation into rabbits, symptoms were not produced until 16 days had elapsed, whereas previously the incubation period had been only 3 days. Negri bodies also occurred although they had not been produced by the strain before glycerolization. These changes, however, passed off with passage.

The method whereby most change has been produced in the characters of *virus fixe* is that of nerve passage. This work has been carried out mainly by continental workers (Kopciowska, 1935 *a*, *b*, Nicolau and Kopciowska, 1934 *b*, 1935 *a*, *b*, 1936). The manner of proceeding is as follows *Virus fixe* is injected into the right sciatic nerve of rabbits The animal dies eventually from rabies, and the left sciatic nerve is then removed and suspended in saline This suspension is injected into the right sciatic nerve of a further rabbit and the performance repeated When this has been carried out on several occasions the strain reacquires some properties characteristic of street virus. The term applied to a strain passed in this way is *ramené en arrière*. The properties of such a strain are as follows·

1. Negri bodies are produced on intracerebral injection of rabbits, and on intracerebral or sciatic injection of dogs
2. The incubation period after intracerebral injection in rabbits is like that of street virus.
3. *Septinévrite* is produced on rabbit injection (see p. 826).
4. Subcutaneous injection of rabbits is noninfective.
5. If a strain *ramené en arrière* is passed by intracerebral injection again it soon reverts to *virus fixe*

The differentiation between street and fixed viruses.

This is a convenient place to discuss the various points in which fixed and street strains have been found to differ

1. On intracerebral inoculation with street virus the incubation period varies from strain to strain and experiment to experiment, but the usual limits are 15 to 21 days. With the conversion to fixed virus that occurs on passage, the incubation period becomes shorter and eventually stabilized at between 3 and 6 days, according to the species of animal

Remlinger and Bailly (1937 *a*, *b*) investigated 11 strains of Tangier street virus, and found that the minimum infective titer varied from 1/2,000 to 1/100,000.

Examining a number of street strains, Mason (1942) found the usual mouse cerebral lethal dose was 10^{3}. Proca and Bobes (1940) regard the limits of infectivity of street strains as extending from 1/500–1/500,000.

Titration of rabies virus in mice. Webster (1939 *b*) showed that rabies virus can be titrated conveniently by intracerebral inoculation of mice. Brain tissue is diluted serially in 10 per cent. horse serum in distilled water, and injected in 0·03 c.c. amounts into 3-week-old W-Swiss mice, using 4 animals per dilution. With increasing dilutions of virus a critical point is reached beyond which less than 50 per cent. of mice succumb. [illegible] as containing 1 MLD. Diluti[illegible] mice. This degree of sensiti[illegible] per gram body weight injected in a similar fashion.

Habel (1940 *a*) has pointed out that up to 0·02 c.c. of an intracerebral inoculum may escape unless special precautions are taken. He recommends that the animal be deeply anesthetized, and the injection made slowly through a short, sharply bevelled 27 gage needle. On withdrawal of the needle, firm pressure should be exerted over the area.

Titration can also be performed if 0.01 c.c. of virus suspension is injected into the lower 1/3 of the gastrocnemius, dilutions of 1/320–1/2560 generally proving fatal (Webster).

In all titration experiments, a more accurate indication of the 50 per cent. end-point is secured by inoculating 6 mice per dilution and working out the calculation by the method of Reed and Muench (1938).

ANIMAL EXPERIMENTS WITH RABIES VIRUS

Routes of Infection

1. *Intracranial* Animals can be most readily infected with rabies by subdural or direct intracerebral injection The technique of subdural injection has been fully described elsewhere (see p. 859), and the intracerebral method only differs in that the needle is actually passed through the soft membranes into the cerebral tissue. Speransky (1927) has adduced experimental evidence that, after subdural inoculation, the cerebrospinal fluid disseminates the virus to all parts of the central nervous system.

The distribution of virus in the central nervous system after intracerebral injection has also been studied After inoculation of dogs with street virus, Remlinger and Bailly (1928 *d*) found virus at the site of injection after 9 days, during the previous 8 days, however, no virus could be found. Working with rabbits injected with street virus, and killing one every day, Remlinger and Bailly (1929 *d*) found that virus was still present locally after 24 hours, but not from the 2nd to 5th days. On the 6th and 7th days virus was found again The virus, therefore, rapidly disappears from the site of inoculation in dogs and rabbits, both highly susceptible to rabies In tortoises, however, which develop no symptoms, the virus may be demonstrable for months (Remlinger and Bailly, 1929 *b*).

Further confirmation of the apparent absence of virus from the cerebral tissue in the early stages of the incubation period was afforded by some observations of Cornwall and Beer (1925–6 *b*) They found that after intracranial injection of rabbits, passage of the virus could not be continued in series if the brain was removed on the 3rd day Whereas, if it was removed on the 4th day or later, passage was readily continued Lépine and Athanasiu (1947) using street virus in rabbits, detected lesions on the 4th day, but virus was not demonstrable until the 8th day.

2 *Intraneural* The fact that rabies could be transmitted by direct intraneural

Brussels strain was considerably more virulent, although it had been initiated from the same source as the Paris strain.

4. *Hanoi strain.* The strain of *virus fixe* used at the institute in Hanoi has rather active properties (Genevray and Dodero, 1936).

Antigenic Structure of Rabies Virus Strains

A number of experiments has been performed, and will be alluded to throughout these pages, in which cross immunity tests have demonstrated the antigenic similarity of various rabies virus strains Strains of *virus renforcé*, street virus, and fixed virus all appear antigenically related. Animals immunized with one strain prove resistant to other strains The rabicidal serum produced by the injection of one virus neutralizes other strains. All that has appeared from these experiments is that the Pasteur Institute (Paris) strain is a more potent immunizing agent than other strains of virus, although individual substrains vary considerably in this respect.

In recent years, however, the antigenic structure of the rabies virus has been investigated by what may prove to be a more delicate technique. Thus, Havens and Mayfield (1932 *a*) described a specific flocculation as occurring between rabies immune serum and both street or fixed virus suspensions. Further (1932 *b*), they found that rabies virus could absorb its specific antibody from antiserum, and they called this reaction "agglutinin-absorption" They investigated the antigenic structure of a number of strains and decided that at least one and probably more antigens were shared by all They suggested that antigenic differences between strains were due to a quantitative variation in fractions common to all strains (1933). It may thus be that subsequent work will show considerable antigenic variation to exist between strains of rabies virus

Infectivity of Rabies Virus Strains

The best recognized method of estimating the infectivity of any strain of rabies virus is the dilution method, serial dilutions of virus being made and tested for infectivity by injection of rabbits, preferably subdurally. In any comparative series of estimations of virus infectivity it is essential to use the same animal and the same route of injection throughout Guinea-pigs, for instance, may prove more susceptible than rabbits, and thus give a reading of greater infectivity Further, although an equivalent number of infective doses may be administered, it is found that far less guinea-pig infected brain is needed to transmit the infection (by the intravenous route) than rabbit brain (Hurst, 1937)

Working on these lines with *virus fixe*, Stuart and Krikorian (1929–30) defined a minimum lethal dose as that dose invariably producing symptoms in a rabbit of 1,400 gm weight 120 hours after subdural inoculation In preparing suspensions for titration careful attention to the diluent is essential, and 10 per cent horse serum in distilled water is probably the most useful The material should be mechanically emulsified

Fixed virus This question is fully discussed in the section on Hoegyes's method of antirabies treatment (see p 861). Here it may be said that at the introduction of this method dilutions up to about 1/5,000 usually proved infective. With the continued passage of certain strains of *virus fixe*, however, the titer has risen very considerably Thus, Remlinger (1935 *a*) found that the Tangier strain was active to a dilution of 1/900,000 Proca and Bobes (1940) found that the Bucharest strain of fixed virus, passed for over 50 years, was infective in brain tissue at 1/10,000 and sometimes up to 1/50,000, cord was infective at 1/10,000

Using Swiss mice, Mason (1942) found that various fixed strains had an average mouse cerebral lethal dose of 10^{-5}

Street virus Tested by the Hoegyes dilution method in experimental animals, it is found that street virus strains vary considerably in their infectivity Thus,

for some weeks. He found that rabbits born even before the appearance of symp-

did not contract rabies, embryos injected with street virus developed symptoms and Negri bodies could be found (de Oliveira and de Bettencourt, 1943).

8. *Rabies virus and experimental tumors* Street virus can proliferate in a Brown-Pearce neoplasm in the eye Further, the rabic infection can be passaged by inoculating pieces of tumor infected *in vivo* into the eye of another rabbit (Schoen, 1937). If tumor is growing in one eye, and street virus is injected intracerebrally or into the other eye, Negri bodies may develop in the tumor cells (Levaditi, Schoen, and Reinié, 1937 *a*). The injection of rabies virus into the lesions of rabbits bearing Shope's papillomatous tumors transmits the infection, and the animals die of rabies, but without any virus being found in the tumor (Levaditi, Schoen, and Rienié 1937 *b*) If rabbits are injected into the anterior chamber of the eye, virus only rarely localizes in the tumor.

9. *Other routes of injection.* Infection can be quite readily transmitted by injection into the tongue of various animals (see Remlinger, 1925 *a*, Reichel and Schneider, 1934, 1936). Injection into the tail is also a possible method of transmitting the infection Mice and rabbits may be infected by this route (Proca, Bobes, and Jonnesco, 1934 *b*). Remlinger and Bailly (1938 *d*) found that rabbits could rarely be infected by exposure to an atmosphere containing desiccated or pulverized virus.

The following routes may also be used intraspinal, intra-ocular, intranasal, dermal, injection into certain mucosae, injection into certain solid organs, and intraperitoneal

General Manifestations of Experimental Rabic Infection

Although many animals are susceptible to rabies, and the manifestations differ somewhat from animal to animal, and strain to strain of virus, there are certain common features

Furious rabies Furious rabies is seen in its classical form in dogs infected naturally or experimentally with street virus Furious symptoms may occur in other animals suffering from street virus infection (e g, guinea-pig and fowl). Furious symptoms, usually of relatively short duration, may be found in certain cases of *virus fixe* infection before the onset of paralysis

Animals suffering from furious rabies are at first restless ...

festations The animals usually rush about their cages in a staggering way, falling down from time to time They show a special tendency to snap and bite, harsh cries or roars are usually ...

of illness is that of para...

animals are found lying ...

out this terminal phase ... after a few days illness, although with certain attenuated strains symptoms may persist for 10 days or so (Remlinger and Bailly, 1942 *b*)

Dumb rabies Dumb or paralytic rabies is found in its most typical form in rabbits infected with rabies, especially *virus fixe*. It is found in rabic infection of many other animals also ...

form Chinese hamster, ...

injected intravenously wi...

injection was shown by di Vestea and Zagari in 1887. They injected dogs and rabbits in the sciatic and median nerves, and secured infection by this means These observations have been widely confirmed, and the possibility of transmitting rabies by direct intraneural injection is now universally accepted (Roux, 1888 *a*, Ferré, 1888, di Vestea and Zagari, 1889, de Blasi and Travalli, 1889, Moon, 1913, Nicolau and Kopciowska, 1934 *c*).

Infection has been transmitted to rabbits, dogs, and guinea-pigs by injection of the sciatic, median, vagus, facial, hypoglossal, sympathetic, and optic nerves Proof that virus actually spreads via the nerve trunks after intraneural injection is afforded by various facts. In the first place, paralysis may first involve the inoculated limb Second, the cord may show the presence of virus at the site of connection of the inoculated nerve. Third, virus cannot be isolated from the blood with any regularity, suggesting that this is not the means of dissemination of the virus. Fourth, virus has been injected intraneurally after transection of the spinal cord Later, virus was recovered from the distal, but not the proximal, part of the cord

The incubation period after neural inoculation varies according to the strain of virus and the nerve injected Thus with street virus the incubation period was about 18 days, and with fixed, 9 days (di Vestea and Zagari, 1887) In a series of experiments by de Blasi and Travalli (1889) the periods varied as follows median or sciatic nerves, 11 to 13 days, facial nerve 6 to 10 days, hypoglossal nerve 8 to 9 days, vagus nerve 7 to 9 days, sympathetic nerves 15 to 18 days

3. *Intravenous.* Rabies virus injected by this route usually produces paralytic rabies (Pasteur *et al.*, 1882). It has been found that infection is most likely to develop if the inoculum is guinea-pig brain, rabbit brain is not so infective (Hurst, 1937).

4. *Combined injection* It is said that if rabbits are injected subdurally with street virus, and during the period of incubation an intravenous injection is given, the infection is negatived and no rabies develops (Puntoni, 1921 *a*).

5. *Intramuscular* Rabbits and other animals can be quite readily infected by injections into the muscles After such an injection virus probably ascends to the central nervous system by nerve fibers The work of Goodpasture (1925) has made it appear very likely that this actually occurs Thus, he carried out a study in which he injected rabbits in the masseter muscle Infection spread to the central nervous system, probably by way of the trigeminal nerve. After such injection paralysis did not occur in the hind limbs, in the front limbs it developed more commonly on the side of the inoculation than on the other side. Histologically, there was usually an extensive cellular reaction affecting the central portion of the sensory division of the 5th cranial nerve and the gasserian ganglion

Hurst (1937) *reported that guinea-pig brain infected with passage virus is* more infective by the intramuscular route than is rabbit brain

6 *Subcutaneous* The subcutaneous route is not one of the most certain for the transmission of rabies. Thus, the dog is relatively insusceptible, especially to *virus fixe*, the rabbit is more susceptible.

Mice can be infected subcutaneously, and Webster (1937), using his specially bred susceptible stock, has found virus in the central nervous system at the site of connection of the nerve supply of the injected area The virus cannot be found until 5 to 6 days have elapsed

7. *Hereditary infection* A number of authors has pointed out that hereditary infection may occur in animals, the virus passing from a mother developing rabies shortly before delivery to her newborn offspring (Perroncito and Carita, 1887, Loir, 1903, Lanfranchi and Lenzi, 1918) Others have failed to confirm this transplacental passage of virus (e g, Zagari, 1888)

Konradi (1905, 1908, 1914, 1916) has made a special study of transplacental passage of virus He found that, in traversing the placenta, the virus loses much of its infectivity, so that animals inoculated with the fetal material should be examined

Routes of infection.

Dogs can be infected by numerous routes and develop furious or paralytic rabies with the other features of illness already described The state of nutrition of the animal does not affect its susceptibility (Remlinger and Bailly, 1932 *a*, *b*), puppies are usually much more susceptible than adult dogs (Remlinger, 1908 *e*). Beagle dogs have been much used in Webster's work.

Intracranial. In antirabies vaccine practice the usual route of injection is the subdural (see p 859). Animals infected by the subdural route are often spoken of as infected by trepanation, or as having been trepanned. The intracerebral route is widely used in experimental work. After intracranial injection with street virus, furious rabies develops in about 15 to 21 days. Fixed virus usually produces paralytic rabies, but sometimes the furious form, the incubation period being usually under 8 days.

Intraneural As we have already mentioned, dogs may be infected by injection into the median, sciatic, or other nerves (see p. 817). The first symptoms usually develop in 10 to 21 days' time, depending on the site of inoculation and the strain of virus

Intra-ocular. Injection into the anterior chamber of the eye usually secures infection (Roux and Nocard, 1890, Remlinger, 1904 *g*). The virus probably spreads to the brain by the optic and other nerves.

Dermal Intradermal injection usually fails to transmit the infection, and it has been reported that superficial scratches rubbed with virus do not become infected (Babès and Vasilu, 1911)

Intramuscular. Injection into muscles such as those of the neck is usually positive, but Marie (1912 *b*) reported that intramuscular injection of the abdominal walls was only positive in 40 per cent. of cases A special application consists in injecting animals in the tongue. As compared with intracerebral injection, 10 times as much virus is needed to infect if the inoculation is made in the tongue, 40 times if in the neck, and 1,000 times if in the muscles of the hind leg (Webster, 1942).

Intravenous. Dogs injected intravenously usually develop paralytic rabies (Pasteur *et al*, 1882, Remlinger and Mustapha Effendi, 1904, Remlinger, 1904 *g*). However, small doses of virus may result in the development of furious manifestations (Pasteur *et al*, 1884)

Intravesical. Dogs have been infected by instillation of the bladder (Remlinger and Bailly, 1938 *f*).

2. Rabbit

Canine rabies was early found to be transmissible to rabbits (see, e.g., Galtier, 1879, 1881, 1890, Pasteur *et al*, 1881 *a*, Raynaud and Lannelongue, 1881, Helman, 1889, Leclainche and Morel, 1899). The susceptibility of the rabbit to rabic infection is of the greatest advantage in experimental work and in antirabies practice. Of particular merit is the factor of safety to the experimenter, as the rabbit practically always develops paralytic rabies, and shows no tendency to bite.

In Tonkin, Dodero (1939) reported a shorter incubation period in infected rabbits in the summer

Routes of infection.

Rabbits can be infected by all the common routes, subdural, intracerebral, intraneural, intra-ocular, dermal, subcutaneous, and intramuscular. They may also be infected by rubbing virus onto a patch of recently shaved skin (Remlinger, 1905 *f*)

that street virus produces furious rabies and *virus fixe* dumb rabies, but there are so many exceptions to this rule that it scarcely applies to animals other than the dog

Animals suffering from paralytic rabies may show a short preliminary phase of excitation, and there may be a tendency to bite throughout the illness. Very soon, if not as the first manifestation, paralysis develops, affecting characteristically the lower extremities. At first the animal is ataxic, but soon it falls to the ground and is unable to rise. The lower jaw is usually paralyzed also. The animals usually waste before death, and polyuria may occur. Albuminuria is very frequent (Auffret and Dodero, 1940 *b*).

Wasting. There has been some discussion regarding the occurrence of wasting as a manifestation of rabic infection. Thus, Lamb and McKendrick (1909) found that certain rabbits injected subdurally with street virus (or subcutaneously with fixed virus) kept in good health for some time, but later became wasted and died with no other manifestation of infection, in certain cases the wasting set in earlier and was more progressive. The virus could only be demonstrated by passage of infected tissue. Remlinger (1927 *d*), however, considered that the existence of such a condition as "consumptive" rabies in animals had not been proved.

Temperature. A number of workers has recorded a rise of temperature in rabies (e.g, di Vestea and Zagari, 1887; Hoegyes, 1888, Babès, 1888, Roux and Nocard, 1890). This rise usually occurs about 6 to 7 days after the inoculation of virus, although it may occur earlier. It is more uniformly present in *virus fixe* infections than with street virus. The rise of temperature is of importance, as it usually occurs concomitantly with the presence of virus in the saliva, and therefore with the potential infectivity of the animal (Nicolas, 1906).

Respiration. Ferré (1888) drew particular attention to an increase in the respiratory rate. This increase begins in the incubation period and persists throughout the illness, except that in the terminal paralytic phase of street virus infection the respiratory rate usually becomes lessened

Blood changes Certain authors have studied the blood counts in experimental rabies and found a polynucleosis (Courmount and Lesieur, 1901, Nicolas, 1903). Nicolas and Bancel (1905) suggested, however, that this rise was nonspecific as it occurred after injection of normal as well as rabic cords.

Biochemical changes The blood urea has been found to rise in rabid dogs and rabbits, and glycemia may occur (Remlinger and Bailly, 1937 *c, d*) There is an increase in the blood chlorides, especially in the cells (Remlinger and Bailly, 1937 *e*).

The serum of rabid rabbits has been found to exert a neutralizing effect on atropine (Tzekhnovitzer and Goldenberg, 1929)

The adrenalin content of the adrenals has been found much diminished in the terminal stages of *virus fixe* infection (Porak, 1912)

Recovery. Animals seldom recover from experimental rabies but there were certain references to this in the older literature (see, e g, Pasteur *et al*, 1882, 1884, Remlinger and Mustapha, 1904, Vincent, 1907, Remlinger, 1907 *c*, 1919 *d*) Johnson (1943) has observed several hundred dogs with experimental rabic infection and has not seen a recovery from furious rabies In some instances, dogs may develop an abortive type of illness from which they recover.

ANIMALS SUSCEPTIBLE TO RABIES VIRUS

1. Dog

Virtually all our modern knowledge of the virus, and of the prevention of rabies, dates from the early experiments of Pasteur and others on the transmission of the infection to the dog (see, e g, Pasteur *et al*, 1881 *b*, 1882, 1884, Pasteur, 1887, Ernst, 1887, Finkelstein, 1887, Gamaleia, 1887 *b*, Helman, 1889, Ferré, 1888, 1889, Marie, 1907 *b*).

(*f*) *Fulminating form* This type of infection is characterized by a rapid and practically asymptomatic death [illegible]

Factors influencing symptomatology.

Virus fixe always gives paralytic rabies, but street virus usually produces one of the other forms The site of inoculation with street virus influences the subsequent manifestations (Remlinger); thus subdural and intracerebral injection usually produce furious rabies Intra-ocular injection gives rise to spasmodic rabies, and nasal inoculation to attenuated furious, dyspneic, or pseudosepticemic rabies. Intramuscular inoculation may give localized tetanic convulsive movements, intravenous injection may produce a flaccid paraplegia

4. Mouse

Mice have been used extensively of recent years, especially in diagnostic work and titration experiments. They are probably about 10 times as susceptible as guinea-pigs or rabbits They can become infected following the bite of a rabid dog or cat, or by ingesting an infected cadaver (Remlinger, 1908 *d*). Mice can be infected experimentally with street or fixed virus by the following routes intracerebral, intramuscular, subcutaneous, intraperitoneal, intranasal, and by injection into tongue or tail, approximately 100 times as much virus is needed to infect by [illegible] subcutaneous route, as compared with the [illegible], Jonnesco, 1934 *b*, Proca, Bobes, and Jon- [illegible] and Leach, 1940 *a*, Remlinger and Bailly, 1940 *b*, 1941 *a*, *b*, Sabin *et al*, 1940, Mason, 1942, Baranovskaya, 1943, Kligler and Bernkopf, 1943). Of recent years Webster has recommended a specially bred susceptible strain of mouse (Webster and Dawson, 1934–5, Webster, 1936, 1937, 1939 *b*, 1942). Any ordinary "laboratory" mouse may, however, be almost equally well used

Age plays an important part in determining susceptibility to rabic infection Casals (1940 *a*) found that 7–9 day old mice were more susceptible than older mice however injected, 20-day-old mice were more susceptible than 60-day to peripheral but not intracerebral injection of fixed virus, 20- and 60-day mice were equally susceptible to street virus (see also Casals and Webster, 1940)

Mice suffering from symptoms due to fixed virus are abnormally susceptible to the effect of glucose injected intraperitoneally, probably because of the resulting dehydration (Hoyt *et al*., 1939).

Clinical features.

The following types of illness may develop in infected mice (Remlinger and Bailly, 1940 *b*, 1942 *c*),

Furious rabies This type is seen chiefly after intracerebral or peripheral injection of street virus. The animal makes rapid movements and jumps about the cage, tending to bite other animals Paralysis may develop These features last from 1–3 days, and death is often sudden After intramuscular injection there may be a tendency to automutilation by biting Another symptom is a marked tendency to scratch

Paralytic rabies After an incubation period of 7–19 days following injection of fixed virus, the animal develops tremors, an unsteady gait, incontinence and definite paralysis, either hemiplegic or ascending The onset is often sudden and these features may not be observed

Other forms Remittent, pseudotetanic spasmodic, atypical fulminating, and spasmodic initial monoplegic types have been described

Rabbits inoculated intracranially with *virus fixe* usually develop paralytic symptoms after 5 days, and are dead by the 8th day, although the disease may sometimes progress even more quickly (Grysez, 1941).

In addition to the routes of infection commonly used in rabies experimental work, certain others have been employed.

Corneal. Rabbits can be infected by corneal inoculation of either street or fixed virus (Kraus and Fukuhara, 1909, Levaditi and Schoen, 1935 *a*).

Ingestion Ingestion of rabies virus was said to prove infective (Galtier, 1881), but the majority of present-day investigators does not believe that infection can be transmitted by this route, at any rate with any readiness.

Injections into the viscera. Levaditi and Schoen (1935 *a*) reported that infection resulted after injection of the parotid gland, adrenal, testis, or ovary.

Nasal. Rabbits can be infected by swabbing the nasal mucosa with virus (Galtier, 1890, Remlinger, 1904 *b*, Levaditi and Schoen, 1935 *a*).

Other mucosae. In the course of their study on the local production of Negri bodies in mucous membranes, Levaditi and Schoen (1935 *a*) found that manifestations of rabic infection followed inoculation of the conjunctival, lingual, tracheal, and intestinal mucosae.

3. Guinea-pig

Guinea-pigs have been fairly extensively used in experimental work (see, e g, Pasteur *et al.*, 1884, Gibier, 1884, Lamb and McKendrick, 1909, Nicolle, 1917, Panisset and Dischamps, 1920, Figueira, 1924, Schweinburg, 1932 *b*, Jacotot, 1937, Virga, 1938, Raynal and Lieou, 1940 *a*).

Remlinger, in particular, has studied experimental rabies of the guinea-pig, and his work is still the most complete study (1917 *a*, *b*, *c*).

Routes of inoculation.

The following routes are in general use. intracerebral or subdural, intra-ocular, nasal, intramuscular, and intravenous The animals may be infected by intracardiac injection *Virus fixe* injected intraperitoneally is usually noninfective, but a preliminary dose of India ink may produce a positive result (Marie, 1932, 1934). Remlinger (1906 *a*) was able to secure infection by injecting lymph glands enlarged by tuberculosis. The mucosae are susceptible to invasion by street virus (Piringer, 1945).

Clinical features.

Remlinger has described the following manifestations of rabies in the guinea-pig

(*a*) *Furious rabies.* The animal's hair bristles, it is excited in its movements, and often rushes about the cage, falling down from time to time, sexual excitement also may be very marked The respirations are accelerated The animals often bite their companions, but not man.

(*b*) *Attenuated furious rabies* The symptoms in this type of infection are as in furious rabies, only very much milder

(*c*) *Dyspneic form* This type of reaction is characterized by little else than a very accelerated respiration rate

(*d*) *Pseudosepticemic form* In this type of illness the animal is laid low by the virulence of the infection without showing evidence of excitation. In certain cases the animal may die suddenly, after some weakness and perhaps slight paralysis, about 3 to 5 weeks after inoculation (Remlinger and Bailly, 1931 *b*)

(*e*) *Spasmodic form.* This type usually follows intra-ocular injection and begins by an intense local pruritus and inflammatory reaction in the eye The animal has pharyngeal spasms and expels saliva With the progress of the infection, convulsions, spasms, and guttural cries occur

occur in the cerebellum where there is perivascular and interstitial inflammation (see also Veeraraghavan and Philipsz, 1940).

Stork. Subdural inoculation of *Ciconia ciconia* was found to give rise to paralytic rabies (Remlinger and Bailly, 1936 *b*).

Pheasant This bird was infected by Jacotot (1938)

Peacock. Jacotot and Le Roux (1938) transmitted infection to a peacock.

Other birds. Canaries, geese, and ducks can also be infected, although it may not be possible to pass the virus in series indefinitely (Marie, 1904 *a*). The sparrow is also susceptible (Veeraraghavan and Philipsz, 1940).

14 *Tortoise.* Many years ago it was shown that the tortoise was refractory to rabies (Remlinger, 1904 *b*, 1905 *b*). More recently, interest has been revived, be- [illegible] remain latent and be [illegible] time (Remlinger and [illegible] 1). Thus, street virus [illegible] *Testudo mauritanica* (Remlinger and Bailly, 1931 g, 1932 *a*). The animals did not, however, show any sign of infection A similar state of affairs obtains in the case of certain other tortoises e g, *Clemmys leprosa*, where virus was recovered, in one experiment, after 150 days (Remlinger and Bailly, 1932 *a*), and *Cynixys belliana tortues* (Mezger, 1932).

15. *Toad and frog.* Unlike the tortoise, virus only survives for a few days after intracerebral injection (Remlinger and Bailly, 1929 *e*, 1930 *g*, *h*, 1932 *a*) The animals prove quite refractory and no symptoms develop After injection into the dorsal lymph sac, virus undergoes a gradual attenuation of virulence (Babès and Cerchez, 1891).

DISTRIBUTION OF VIRUS IN INFECTED ANIMALS

Site of Inoculation

In normal mice and guinea-pigs, street virus introduced peripherally remains viable in the muscle for at least 4 days, and invades the nervous system within 24–48 hours (Habel, 1941 *b*).

Central Nervous System

Brain and cord After intracranial injection, virus is to be found locally for a comparatively few hours only. During the next 3 to 4 or more days no virus can be found at the site of inoculation, but reappears thereafter. During the first few days after injection the virus is spreading to the bulb, cord, and peripheral nerves, but without causing any symptoms, although there may be a rise of temperature (see also p 810) A number of authors has recorded the presence of virus in the bulb of animals injected by various routes during this incubation period (di Vestea and Zagari, 1887, Gamaleia, 1887 *c*, Roux, 1888 *a*, Remlinger, 1905 *c*, *d*, Levaditi, Nicolau, and Schoen, 1924 *c*, 1926, Palawandow and Serebrennaja, 1931 *b*).

After intracerebral injection, street virus has been found in large quantity in the facial nucleus 5 days before the onset of symptoms (Remlinger and Bailly, 1928 *d*) With the onset of symptoms it is found that virus has spread to all parts of the central nervous system, although in the dog certain parts may be found avirulent, the distribution being rather irregular (Koch, 1929, Remlinger and Bailly, 1931 *a*).

After neural injection virus can first be isolated from the spinal cord in the area associated with the inoculated fibers (di Vestea and Zagari, 1889). At a later stage in the disease, the virus can be isolated from all parts of the cord. After injection by other routes also, virus can be isolated from the cord, as from other parts of the central nervous system For example, after intramuscular injection (gastrocnemius) in mice, virus appears in the lumbar cord 3–5 days later and passes

Histological lesions occur in the brain and cord, and in the posterior root ganglia after peripheral inoculation.

Other Animals

5. *Monkey.* Monkeys can be infected subcutaneously with *virus fixe* (Helman, 1889; Lamb and McKendrick, 1909), also subdurally, intramuscularly, and intraperitoneally. Fixation of street virus can be secured by repeated subdural passage in monkeys (Acton and Harvey, 1912–13). Monkeys can be readily infected with street virus by injection of the neck muscles.

6. *Cat.* Cats can be infected by subdural or intra-ocular injections, but have not been used extensively in experimental work; the symptoms may be furious or paralytic.

7. *Fox.* França (1905 *b*) transmitted street virus to the fox by intramuscular injection; the disease assumed either the furious or paralytic form.

8. *Rodents.* Gibier (1884) found that rats were susceptible, and Remlinger (1904 *c*) infected rats and mice. França (1905 *a*) found that the following members were susceptible. *Mus decumanus, Mus rattus, Mus musculus,* and *Arvicola incertus.* Fermi (1907) found that rats and mice were especially susceptible to Sassari fixed virus.

Rat. Rats are susceptible to infection with rabies virus by the ordinary routes of infection, except that they are said to be comparatively insusceptible to subcutaneous injection of street virus, although younger animals are more susceptible than older (Legezynski and Markowski, 1928 *b*).

The Chinese hamster can be infected, both with street and fixed viruses, by intracerebral injection (Yen, 1936 *a, b*). The resulting disease is usually paralytic and Negri bodies are found.

[illegible]) which was [illegible] bite, and it [illegible] and Negri bodies were present (Vianna, 1929).

9. *Horse.* The horse can be infected experimentally with rabies, and this has been reaffirmed by Jacotot and Le Roux (1937). Infection can be transmitted by the intracerebral route and paralytic rabies develops, there may, however, be some signs of excitation and attempts to rise.

10 *Calf* Calves may be infected by intracerebral injection and develop paralytic rabies (Lewthwaite, 1930, Jacotot and Le Roux, 1937)

11. *Sheep* This animal can be infected with rabies quite readily.

12. *Hedgehog.* Hedgehogs are susceptible to both street and fixed viruses (Jonnesco, 1927 *a*, 1932 *a*) After subdural inoculation paralysis develops in 7 to 8 days, the animal may have convulsions and tetanic spasms, and attempt to bite *Passage of virus fixe* through hedgehogs increases its virulence for rabbits, as well as dogs, which may develop furious symptoms

13. *Birds. Hens and cocks* can be quite readily infected with rabies and develop paralytic manifestations (Gibier, 1884, Pasteur, Chamberland, and Roux, 1884, Remlinger and Bailly, 1929 *a, f*, 1930 *d*, Veeraraghavan and Philipsz, 1940). Sometimes, after intracerebral injection, there may be a stage of furious rabies, terminating in paralysis, recovery may sometimes occur Microscopically, the main changes are to be found in the cerebellum, there is an inflammatory reaction near the Purkinje cells, with perivascular cuffing In the cord, hemorrhages and leukocytic infiltrations are found dorsally Negri bodies may occur in the cerebrum

Pigeon Rabies of the pigeon has been studied in particular by Remlinger and Bailly (1929 *c, f, g*, 1930 *e*), who used street virus Infection is best carried out by the intracerebral route and results in the development of paralytic rabies after about 10 to 24 days, there are disorders of equilibration and flight, histologically, changes

occur in the cerebellum where there is perivascular and interstitial inflammation [illegible]

Peacock. Jacotot and Le Roux (1938) transmitted infection to a peacock.

Other birds. Canaries, geese, and ducks can also be infected, although it may not be possible to pass the virus in series indefinitely (Marie, 1904 *a*). The sparrow is also susceptible (Veeraraghavan and Philipsz, 1940).

14. *Tortoise.* Many years ago it was shown that the tortoise was refractory to rabies (Remlinger, 1904 *b*, 1905 *b*). More recently, interest has been revived, because it appears that after intracerebral inoculation virus may remain latent and be recovered from the brain in a living condition after a long time (Remlinger and Bailly, 1929 *e*, 1932 *e*, Remlinger, Manouélian, and Bailly, 1931). Thus, street virus was recovered 302 and 285 days after intracerebral injection of *Testudo mauritanica* (Remlinger and Bailly, 1931 *g*, 1932 *a*) The animals did not, however, show any sign of infection. A similar state of affairs obtains in the case of certain other tortoises: e g, *Clemmys leprosa*, where virus was recovered, in one experiment, after 150 days (Remlinger and Bailly, 1932 *a*); and *Cynixys belliana tortues* (Mezger, 1932).

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Central Nervous System

Brain and cord After intracranial injection, virus is to be found locally for a comparatively few hours only During the next 3 to 4 or more days no virus can be found at the site of inoculation, but reappears thereafter. During the first few days after injection the virus is spreading to the bulb, cord, and peripheral nerves, but without causing any symptoms, although there may be a rise of temperature (see also p. 820) A number of authors has recorded the presence of virus in the bulb of animals injected by various routes during this incubation period (di Vestea and Zagari, 1887, Gamaleia, 1887 *c*, Roux, 1888 *a*, Remlinger, 1905 *c*, *d*, Levaditi, Nicolau, and Schoen, 1924 *c*, 1926, Palawandow and Serebrennaja, 1931 *b*).

After intracerebral injection, street virus has been found in large quantity in the facial nucleus 5 days before the onset of symptoms (Remlinger and Bailly, 1928 *d*). With the onset of symptoms it is found that virus has spread to all parts of the central nervous system, although in the dog certain parts may be found avirulent, the distribution being rather irregular (Koch, 1929, Remlinger and Bailly, 1931 *a*)

After neural injection virus can first be isolated from the spinal cord in the area associated with the inoculated fibers (di Vestea and Zagari, 1889). At a later stage in the disease, the virus can be isolated from all parts of the cord. After injection by other routes also, virus can be isolated from the cord, as from other parts of the central nervous system. For example, after intramuscular injection (gastrocnemius) in mice, virus appears in the lumbar cord 3–5 days later and passes

quickly to the brain (Webster, 1939 *b*). Working with mice injected intraperitoneally, Kligler and Bernkopf (1043) found virus in the upper cord after 72 hours, after subcutaneous inoculation in the hind leg virus was first found in the lumbar and thoracic cord, while after inoculation in the fore leg it appeared first in the cervical cord. At a later stage, virus was found in all parts of the CNS.

Cerebrospinal fluid. There is some question as to whether the cerebrospinal fluid may contain the virus or not. It seems that unless cerebrospinal fluid is obtained by puncture, and not during the removal of the brain at autopsy, nerve tissue may contaminate the fluid and give a false positive result (Kerbler, 1933).

Following subdural inoculation, there would seem to be no doubt that virus is disseminated to the central nervous system by the cerebrospinal fluid (see p 828), but it is uncertain as to how long the fluid remains infective.

Septinévrite. This term has been used by French-speaking authors to signify the general dissemination of rabies virus throughout the whole nervous system after injection into any one part of it, whether central or peripheral (Nicolau, 1928, Nicolau and Serbanescu, 1928, Nicolau and Galloway, 1928; Nicolau, Dimancesco-Nicolau, and Galloway, 1929, Nicolau and Kopciowska, 1934 *c*). *Virus fixe* does not produce such general dissemination of *septinévrite* as constantly as does street virus.

After intracerebral injection, a street strain can be isolated from the brachial nerves on the 5th day, and the sciatics by the 6th day, it can also be isolated from the small subcutaneous nerve fibers

The spread of virus throughout the nervous system can be demonstrated, not only by finding the virus in the nerves, but also by the occurrence of specific histological changes The reaction is an interstitial neuritis with infiltration of mononuclears and plasma cells, perivascular cuffing occurs.

Further proof of this general spread of virus throughout the central nervous system has been afforded by the study of injections into animals in which certain nerve paths have been sectioned. Thus, virus was injected into the sciatic nerve after transection of the cord Virus was later isolated from the distal part of the cord and the contralateral sciatic nerve, but not the proximal cord. This clearly showed that nerve paths were the direct means of spreading the virus Further, the cerebrospinal fluid did not appear to play any part (di Vestea and Zagari, 1889)

Another line of approach to this problem was to cut the sciatic nerve shortly after its emergence from the spinal column, and then inject virus intracerebrally. Virus was not isolated from the nerve peripheral to the section, but it occurred in the contralateral nerve (Nicolau and Serbanescu, 1928, Nicolau and Mateiesco, 1928, Nicolau, Dimancesco-Nicolau, and Galloway, 1929).

Other Organs

Blood There has been some difference of opinion over the infectivity of the blood. Thus, certain workers have failed to isolate the virus therefrom (see, e g, di Vestea and Zagari 1887, Helman, 1889). On the other hand, quite a number of workers has isolated virus in both fixed and street virus infections (see, e g, Pasteur *et al.*, 1881 *a*, 1884, Marie, 1905 *b*, Schweinburg, 1932 *a*)

Marie and Urbain (1931) injected rabbits with *virus fixe* by trepanation In the early days of the incubation period, flour, gonacrine, or tuberculin was injected into the subarachnoid space These injections had the effect of making virus appear in the blood

Remlinger and Bailly (1939 *b*) have isolated virus from ticks infesting infected rabbits

Eyes Virus can be found in the retina, choroid, vitreous and aqueous humors (Courmont and Nicolas, 1903, Remlinger and Bailly, 1940 *c*).

Saliva. A number of authors has studied the infectivity of the saliva in dogs

infected naturally or experimentally (e.g., Galtier, 1879, Pasteur *et al.*, 1882, 1884, Roux and Nocard, 1890, Nicolas, 1906, Cruickshank and Wright, 1914). Virus may be isolated from the saliva 3 to 4 days before the onset of symptoms, as well
found 7 days before the onset
may continue to be excreted
ux (1903) was able to show
virus has been isolated from
the submaxillary and other salivary glands, it is highly probable that the virus is excreted by these organs (Raynaud and Lannelongue, 1881, Pasteur *et al.*, 1884; Poor, 1906; Cruickshank and Wright, 1914). The characteristic hydrophobic froth is probably noninfective (Remlinger, 1928 *b*), saliva produced in response to injection of pilocarpine proves noninfective (Remlinger, 1904 *e*). Rabbit saliva may prove infective (Pasteur *et al.*, 1881 *a*).

Lacrimal glands. Virus has been isolated from the lacrimal glands in experimental rabies of dogs and rabbits (Tscheschkow, 1931) It has also been isolated from the lacrimal gland of Harder in rabbits infected with *virus fixe*, and dogs infected with both street and fixed viruses (Remlinger and Bailly, 1938 *c*).

Mammae. Bardach (1887 *b*) found virus in the mammary tissue and milk of lactating rabbits The milk of infected guinea-pigs may rarely contain the virus (Remlinger and Bailly, 1932 *a, d*).

Liver. The presence of virus has been recorded in the liver (Remlinger and Bailly, 1931 *a, b*; Schweinburg, 1932 *a*)

Kidneys. This organ may contain virus (Remlinger and Bailly, 1931 *a, f, b*)

Adrenals

Spleen

and Bailly,

Pancreas

Lungs. Bert (1878) examined rabid dogs and reported that the pulmonary secretion was probably infective. Infectivity has been found by Remlinger and Bailly (1934 *a*), examining rabbits, dogs, guinea-pigs, and cats

Testes. These organs contain the virus only on rare occasions (Schweinburg, 1932 *a*).

Lymphatic system. Virus was found in the mesenteric, axillary, and inguinal glands a short time after intraperitoneal injection of mice and occasionally reappeared in the mesenteric glands after some days (Kligler and Bernkopf, 1943).

Autosterilizable Neuro-infections

This term has been applied by French authors to a condition where no virus can be isolated from an organ almost certainly infected It is supposed that the reaction of the host is so efficient that the virus is destroyed and cannot be isolated. The existence of this condition has been postulated in rabies as in other neurotropic virus diseases (Levaditi, Sanchis-Bayarri, and Schoen, 1928, Remlinger, 1928 *c*, and others)

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virus (Nicolau, Mathis, and Constantinesco, 1933) After neural inoculation in rabbits, the cord may show an intense reaction without the presence of virus,

th

from a supposed case of autosterilizable neuro-infection, guinea-pigs and mice should be injected, owing to their greater susceptibility.

PATHOGENESIS OF EXPERIMENTAL RABIES

Portal of entry Animals can be infected by a variety of routes, but most readily by those in which virus is applied more or less directly to nerve fibers. Thus, intracranial and intraneural injections regularly secure infection. Animals can also be infected by the dermal, subcutaneous, and intramuscular routes, and by injections into the tongue or tail After injections by these methods also it is presumed that virus spreads to the central nervous system by nerve fibers.

Spread of the virus. The spread of virus in the nervous system has already been fully discussed (*septinévrite*, see p. 826). Thus, after both intraneural and intracerebral injection, virus is disseminated to all parts of the central and peripheral nervous system. Its presence may be detected by animal inoculation tests, and by the occurrence of histological lesions, including Negri bodies in the endoneurocytes

Further proof of the rôle of the actual nerve fibers in the spread of rabic infection has been afforded by experiments in which the spinal cord was cut. After *injection of the sciatic nerve, virus could be found only in the distal part of the* cord and the contralateral nerve. Also, when virus was injected intracerebrally after section of the sciatic nerve, virus was not found distal to the cut, but was present in the contralateral nerve.

Yamaki (1937) injected rabbits intravenously with virus, and studied chronaxia of various muscle groups His studies led him to postulate a neural spread of virus, as signs of nervous irritation were evident during the prodromal period

These experiments showed that it was unlikely that the cerebrospinal fluid playe[illegible] injection. [illegible] agent [illegible] somewhat different after subdural injection. Here the work carried out by Speransky (1927) seems to show convincingly that virus is disseminated to other parts *of the central nervous system* by the cerebrospinal fluid, as indeed *might reasonably* be expected This author found that when cerebrospinal fluid was drawn off prior to subdural injection, the incubation period was prolonged Also, by a special technique, he was able to infect the surface of the brain without the needle passing through the cerebrospinal fluid In such a case the incubation period was again prolonged Finally, he showed that intraspinal injection of antiserum neutralized subdural inoculations of virus made a short time earlier or later.

The possible rôle of the blood has still to be considered Is virus disseminated through the body by the blood? That this is unlikely is suggested by the comparatively few occasions on which virus has actually been isolated from the blood Schweinburg and Windholz (1930) have investigated the rôle of the blood by joining two rats together by the skin One animal only was infected with rabies and eventually died therefrom. The other animal did not contract rabies This *experiment supports the opinion that the virus does not spread* by the blood, as presumably there was some vascular communication between the two animals Although to postulate spread by the blood does not prove attractive, this route would appear to operate in those cases of hereditary rabies developing in the newborn of infected mothers, some being in the incubation period and not actually rabid (see p. 818)

Primary tissue reaction With regard to the primary tissue reaction in rabies, no series of experiments quite comparable to that of Hurst in poliomyelitis (Ch LXXXIV) appears to have been carried out, although certain observations have been made.

A series of experiments was carried out by Laignel-Lavastine and Voison (1906) in which they injected rabbits intracerebrally, killing one every day They found that on the 2nd day the nerve cells of the cord showed only some nuclear chromatolysis For the next few days changes were those of swelling, distortion, and

eventual disappearance of the Nissl bodies, and vacuolation of the cytoplasm Lastly neuronophagia was found by the 6th day.

Goodpasture (1925) carried out a series of experiments in which rabbits were injected in the masseter muscle. The first evidence of injury to the nervous tissue was detected in the nerve cells of the gasserian ganglion. It was found that these cells might become completely necrotic without there being any evidence of cellular reaction in the surrounding tissue.

It appears, therefore, that the primary tissue reaction in rabies is a primary neuronal degeneration, as in poliomyelitis

PATHOLOGY OF EXPERIMENTAL RABIES

Central Nervous System

[illegible]

infections Further, there may be a tendency for the reaction with *virus fixe* to be more cellular.

The meninges of the brain and cord are usually edematous and hyperemic and may be infiltrated with mononuclear cells Minute hemorrhages are a characteristic feature of rabic infections, and occur perivascularly and diffusely throughout the brain and cord, perivascular "cuffing" is another feature, but of somewhat variable intensity. The epithelium of the central canal may show proliferation, and hemorrhages may surround the canal Microgliosis may be found in certain cases Demyelination does not occur (Grinker).

Nerve cell changes Degenerative changes are usually found in the anterior horn cells and may also be noted in the Purkinje cells Changes in the neurofibrilliary apparatus have attracted some attention (Cajal and Garcia, 1904, França, 1905 *b*, Marinesco and Draganesco, 1932, Manouélian, 1935 *c*, 1936 *a*) These changes occur in infections with both fixed and street virus and take the form of overstaining, hypertrophy, and fusion into thick cords, a plexus-like formation may eventually develop Manouélian (1935 *c*) drew attention to these changes, particularly in the spinal ganglia, but they occur also in the nerve cells of the bulb, Ammon's horn, vagus nucleus, and spinal cord These neurofibrillary alterations may be very prominent in the nerve cell in the centre of a rabic nodule, even though the cytoplasm and nucleus of the cell have disappeared (Manouélian).

Changes have also been described in the mitochondria, Golgi apparatus, and Nissl bodies. Thus, Tupa (1929) reported that the nerve cells of the rabbit's brain infected with *virus fixe* showed poorly staining mitochondria, poorly stained and hypertrophied Golgi apparatus, and fragmentation of the Nissl bodies.

Babès nodules These lesions have already been described, and the lesions in animals resemble exactly those of the human disease (Babès, 1892, Germano and Capobianco, 1895, Anglade and Chocreaux, 1902, França, 1905 *b*). Nerve cells are surrounded by a nodule of microglial and mononuclear leukocytes, in severe cases neuronophagia occurs These changes are found in the bulb and to a lesser extent elsewhere in the central nervous system, and occur in both street and fixed virus infections

Van Gehuchten and Nelis nodules These nodules have already been described under the section on human pathology, and their appearance in animals does not differ (Anglade and Chocreaux, 1902, Manouélian, 1903, França, 1905 *b*). Nerve cells in the spinal ganglia are surrounded by proliferated capsular and leukocytic cells, there is also an interstitial inflammatory reaction with mononuclear infiltra-

tion In the severer lesions neuronophagia occurs. Van Gehuchten lesions occur in both street and fixed virus infections.

Other changes. Experimental rabies induces a definite alteration in the mineral constituents of the rabbit brain. Romenski (1935) found that the sodium was diminished and the calcium increased, in the cord the calcium, magnesium, and potassium were increased.

Globules of glycogen may be found in the nerve cells in the bulb and Ammon's horn and in other parts of the central nervous system (Jonnesco and Teodasiu, 1929).

Other Parts of the Body

Salivary glands. Histological changes in these glands are to be expected, as the saliva is usually infective and forms the means of transmitting the causal agent, they have been described by certain authors (Bosc, 1903 *b*, Babès and Jonnesco, 1909 *a*, Manouélian, 1914). The acinar cells usually show degenerative changes, and there is infiltration of the parenchyma and interstitial tissue with leukocytes and macrophages, adenomatous lesions have also been noted.

Mucous membranes. Manouélian and Viala (1928 *a*) have described microscopical lesions of the mucosae of the mouth and tongue; these take the form of minute abscesses [illegible] cells

Muscles. Loss [illegible] have been recorded in rabid [illegible]

Spleen Lesions have been described in the spleen in both street and fixed virus infections (Babès and Jonnesco, 1909 *b*). Thus, the follicles are hypertrophic, hyperemic, and hemorrhagic, and there may be degeneration of certain of the central cells

Kidneys. Changes are said to occur in the kidney in rabic infections of experimental animals (Babès and Jonnesco, 1909 *c*). The glomeruli are congested and may show hemorrhages, they may also be infiltrated with fibroblasts, mononuclears, and even polymorphs There is some cellular infiltration of the interstitial tissue also.

Adrenals. Marked karyorrhexis has been reported in the adrenals of animals infected with *virus fixe* (Nicolas and Bonnamour, 1905).

Testes Babès (1909 *b*) found that spermatogenesis was active in dogs suffering from furious rabies, but diminished in paralytic rabies

CAUTERIZATION IN EXPERIMENTAL RABIES

A number of workers has explored the effect of cauterization on wounds experimentally infected with rabies virus Thus Babès and Talasescu (1894) infected wounds of dogs or rabbits with fixed virus, and cauterized with the thermocautery If the wounds were cauterized more than 25 minutes after infection, rabies developed Cabot (1899), on the other hand, who infected guinea-pigs intramuscularly with fixed virus, found that when the track of the needle was treated 24 hours later, rabies could be prevented, fuming nitric acid and the cautery being more effective than silver nitrate Nitric acid was also found effective in experimental work with guinea-pigs by Poor (1911) and Rosenau (1935), and Jordan *et al* (1939–1940) claimed that ultraviolet light was of value.

The most recent work is that of Shaughnessy and Zichis (1943) who infected guinea-pigs intramuscularly with fixed virus They found that treatment applied within 30 minutes was definitely more effective than when applied after 6 hours They showed that irrigation with soft soap was just as effective as the highly destructive cauterization with nitric acid. Tincture of iodine also proved valuable.

THERAPY OF EXPERIMENTAL RABIES

A variety of chemotherapeutic drugs has been tried in experimental rabies (fixed virus), but without effect e g, plasmoc'
pyridium (Hoyt, Fisk, and Thienes, 1935).
ineffective (Bruni and Buda, 1939, Kirk, 193
Sparteine sulfate has also been used without
Bailly, 1932 *a*, *c*, Hoyt, Fisk, and Thienes, 1935)
to 43° C for 15 to 20 minutes on several occasions during the incubation period did not prevent a fatal result (Remlinger, 1906 *d*) The administration of anesthetics does not influence the illness resulting from rabic infection (Remlinger and Bailly, 1932 *a*, Sulkin, Zarafonetis, and Goth, 1946) The therapy of experimental rabies by antirabic sera has proved quite efficacious and is fully discussed below (see p 851) Antirabies treatment may also be effective (see p 849).

OTHER PROPERTIES OF THE RABIES VIRUS

Morphology, Filtrability, and Size

Elementary bodies have not been much studied, although they have been reported in brain tissue purified by ultracentrifugation (Nicolau, 1939), and have been demonstrated in various tissues by fluorescence microscopy (Gerlach, 1938). Neujean (1945) claims to have demonstrated small "corpuscles" in the blood in rabic infection

The classical inclusion body is of course the Negri body, and this is described in a special section, together
bodies have been described in
lar inclusions in the vasculai
viruses French workers have described the formation of hyperchromatic intranuclear inclusions in nerve cells external to Ammon's horn in rabbits (Levaditi, Lépine, and Schoen, 1929, Lépine and Sautter, 1935). These structures are oval or round in shape and are usually found in the interior of a degenerate eosinophil nucleus They may be found in animals inoculated with phenolized vaccine (Lépine and Sautter, 1938)

The rabies virus (both strains) has been shown to be filtrable by numerous authors (e g, Remlinger, 1903 *a*, *b*, 1904 *a*, Remlinger and Riffat, 1903 *a*, *b*, Marie, 1903, Bertarelli, 1905, Neumann and Mironesco, 1913, Marie and Urbain, 1930, Sato and Kodama, 1931; Funayama, 1935).

It has been found to pass the following ordinary types of filter Berkefeld (V, N, W), Mandler; and Chamberland (L_1 and L_2). Suspensions for filtration should be prepared in broth and made dilute, otherwise difficulty may be experienced.

Levaditi and Nicolau (1923 *a*) reported that rabies virus was able to pass through collodion membranes Later, after the introduction of the collodion membrane ultrafiltration technique for the measurement of viruses, Galloway and Elford (1936) reported the size of fixed virus to lie between 100 and 150 mμ In the hands of Yaoi, Kanazawa, and Satc
0 15 μ by the same method
was slightly higher, 0 14 to c
it is probable, however, that street and fixed virus particles are of the same size, and that the reported differences are due to varying virulence of the filtrates used for inoculation of animals (Lépine and Sautter, 1941)

Cultivation

Tissue cultivation Certain preliminary attempts were made to culture the virus in association with tissue cells, but these experiments did not yield convincing

results (Noguchi, 1912, 1913; Levaditi, 1913). Stoel (1930) reported more suggestive results A number of more recent workers has shown that street and fixed virus can be cultivated in tissue culture (Kanazawa, 1936, 1937, Webster and Clow, 1936, 1937; Bernkopf and Kligler, 1937; Schultz and Williams, 1937–8, Kligler and Bernkopf, 1938 *a*, 1941 *a*, Webster, 1938, Oliveira, 1942, Plotz and Reagan, 1942; Parker and Hollender, 1945).

The virus can be propagated in Maitland's medium. It can also be grown in embryo rabbit, rat, mouse, or chick brain, and in embryo chick tissue, when serum, Tyrode, or plasma is used as the suspending fluid. Under these conditions, virus retains its infectivity for experimental animals.

Veeraraghavan (1945 *a*, *b*, 1946 *a*) has made the important claim that the virus can be grown in a cell-free medium consisting of steamed sheep-brain extract, sheep serum, glycine, and peptone; cultivation under anaerobic conditions was more favorable, growth was favored by the addition of accessory factors (see also 1947 *a*, *b*).

The egg. There has been a number of unsuccessful attempts to infect the embryo and its membranes (Schultz and Williams, 1937–8; Veeraraghavan and Philipsz, 1938, Rita, 1939, Savagnone, 1939). Others, however, have reported changes in the chorio-allantois, or invasion of the embryo without membrane changes (Peragallo, 1937, Kligler and Bernkopf, 1938 *b*, 1939; Bernkopf and Kligler, 1940; Oliveira, 1942, Sigurdsson, 1943). Chick embryos can also be inoculated intracerebrally or intra-ocularly, the infection is of a low-grade type, and the infectivity for animals may become reduced, Negri bodies are readily found (Dawson, 1939, 1941, Sigurdsson, 1943, Parker and Hollender, 1945)

Autolysis

Infected brain preserved in a
to the presence of proteolytic
and preserves infectivity for a l
Virus was recovered after 4 months from autolyzed brain kept at 18° C, but only after a few days when the brain was kept at 37° C. (Ionescu, 1943).

Rabies Toxin

That there might be such an agent as a rabies toxin was suggested many years ago (see, e.g., Remlinger, 1904 *d*), this hypothetical substance has been considered as a possible cause of neuroparalytic accident after antirabies treatment (see p. 884)

Undoubtedly, filtrates of infected nervous tissue may prove toxic to animals, but a similar property can be demonstrated in normal brain tissue (Marie, 1902). Although an acid albuminoid may be extracted from rabic brains which has a toxic effect on animals (Marie, 1912 *a*), there is no evidence for the existence of a true rabies toxin.

Diffusibility

Virus diffuses out of infected brain tissue placed in Ringer-Locke solution into the surrounding liquid (Remlinger, 1917 *d*) A similar diffusion occurs in glycerol (see p. 833).

Reaction to Physical Agents [1]

Cataphoresis Nicolau and Kopciowska (1930 *b*) found the virus to be electronegatively charged (at pH 7 4–5 8) Rabies virus (street or fixed) was also found to be electronegatively charged (pH 7 38) by workers in India, who were able to

[1] With regard to certain of the properties of *virus fixe*, it has been found more convenient to discuss these fully in the section on antirabies treatment, as most of the experiments were carried out with the express purpose of preparing a suitable vaccine

separate virus from the nervous tissue in this way (Sankaran, Iyengar, and Beer, 1934, McCarrison, Sankaran, and Beer, 1934). Working with an *oulou fato* strain, Nicolau, Mathis, and Constantinesco (1933) found this also to be negatively charged

Ultrapressure A strain of *virus fixe* was destroyed at 5,000 atmospheres (Basset *et al*, 1935).

Irradiation. Rabies virus is sensitive to the photodynamic action of methylene blue even in low concentrations of the dye, this sensitivity is not appreciably reduced by the presence of living cells (Shortt and Brooks, 1933–4, Galloway, 1934) It is destroyed by ultraviolet light (Sankaran and Beer, 1934–5 and see p 835), but not by the x-rays (Danysz, 1906). Virus is destroyed by radium emanations (Tizzoni and Bongiovanni, 1906).

Heat and cold The virus is certainly destroyed by heating to 60° to 70° C. (Babès and Lepp, 1889), and usually to 50° C. for 15 minutes (Semple, 1911). At 37° C. virus can remain active, in normal saline, for over 24 hours, and at 20° C. for about a fortnight (Semple, 1911). The virus can be preserved by freezing, and this forms a convenient method of storage (see Remlinger and Bailly, 1942 *d*).

Desiccation Street virus, of course, loses its virulence when desiccated by the Pasteur method, just as does *virus fixe* (see p 859). Bablet, Advier, and Souchard (1927) found that 7-day cords infected with street virus were virulent, 9-day cords attenuated, and 10-day cords nonvirulent. These results agree with the general experience of the reaction of fixed virus to desiccation, although it must be remembered that many strains now show a greatly increased susceptibility (see p 856). Rapid desiccation *in vacuo* preserves the virus and provided no moisture is present, virus remains active even in the presence of air (Remlinger and Bailly, 1940 *a*, *b*) Remlinger and Bailly (1938 *e*) found that desiccated brain infected with street virus survived for 2 minutes at 105° C., 3 minutes at 104° C., and 4 minutes at 103° C.

Reaction to Chemical Agents

Glycerol Generally rabies virus retains its infectivity in 50 per cent. glycerol in the refrigerator for about a year, although street virus may become inactive in a very much shorter time (Remlinger, 1923 *d*, Oliveira, 1942). Partly desiccated cords lose their remaining virulence in glycerol (see p 860).

Bablet, Advier, and Souchard (1927) found that 2-day desiccated cords (street virus) were still virulent after 20 days in glycerol, while 5- and 6-day cords were attenuated after 10 days in this agent The power to produce Negri bodies may be lost before infectivity (Levaditi, Nicolau, and Schoen, 1926). The virus usually diffuses out from the tissue into the surrounding liquid, and may even infect a neighboring healthy brain (Remlinger, 1918 *c*, *d*, 1919 *a*) Dodero (1938 *a*) recorded 2 experiments where treatment with glycerol rendered apparently inactive material virulent, similar observations have been made with herpes virus.

Ether Street virus in infected brain is destroyed in ether in 120 to 125 hours, resembling the findings with *virus fixe*. Street virus was found to be more resistant to ether than Kasauli *virus fixe* (see p 86[illegible]) [illegible]

to ether

Other chemical agents. Both strains of virus are, of course, rapidly destroyed by phenol Formol (1 per cent) also destroys street virus in about 24 hours (Gallea, 1930). Acetaldehyde (½ per cent), benzol, and furfurol all destroy rabies virus in under 3 hours (Cumming, 1914). Sublimate (0.1 per cent), potassium permanganate (0 25 per cent.), and alcohol (50–90 per cent) destroy the virus (Celli, 1886–7). Other lethal agents are soda (0 1 per cent), sodium carbonate (2 per

cent.), hydrochloric acid (0.1 per cent.) and acetic acid (Marie, 1907 *c*). Animal charcoal adsorbs and destroys the virus (Levaditi and Nicolau, 1923 *b*). Sodium ricinoleate destroys the virus (Violle and Livon, 1936; Remlinger and Bailly, 1936 *a*). Ascorbic acid has been found to inactivate fixed virus *in vitro* (Amata, 1937). Fixed virus is inactivated by mustard (Tenbroeck and Herriott, 1946). Fixed virus was found to be resistant to urea (Hoyt and Warner, 1940).

Sulfonamides. These drugs are ineffective in destroying rabies virus *in vitro* (Bruni and Buda, 1939).

Diluents. Milam (1939) examined the effect of various commonly used diluting fluids and found serum-Tyrode solution the best, while serum water was also suitable. Broth, saline, and distilled water are not to be recommended if the other fluids are available.

Reaction to Biological Agents

Rabies virus is destroyed by the lipase in pancreatic secretion, but usually resists trypsin and diastase (Hirano, 1934; Jonnesco, 1936 *b*, see also Bailly, 1945).

Bile neutralizes the rabies virus (Vallée, 1899, Galavielle and Aoust, 1901; *Lesieur*, 1906).

It was found that rabies virus placed in sacs in the peritoneal cavity of dogs and rabbits was destroyed rapidly (Remlinger, 1905 *b*).

Purification

The virus may be purified by dialyzing a glycerol extract through collodion sacs (Poor and Steinhardt, 1913), or by adsorption to kaolin in a neutral or weakly acid medium and elution in a strongly alkaline medium (Sato and Kodama, 1931). It has already been mentioned that virus can be separated from nervous tissue by cataphoresis Virus can also be purified by iso-electric precipitation of infected material (Behrens *et al.*, 1939). The need for purification can, however, be avoided by growing the virus in tissue culture (*vide supra*)

Relationship to Other Infective Agents

Although there would appear to be little or no connection between pemphigus and rabies, it has been claimed that the serum of pemphigus cases destroys rabies virus *in vitro* (Schweinburg and Wolfram, 1937).

Animals immunized against rabies showed no immunity to vaccinia, and the sera of variola cases did not neutralize rabies virus (Dodero, 1940 *a*).

No cross immunity could be demonstrated between Venezuelan equine encephalomyelitis and a fixed rabies strain of bovine origin (Kubes and Gallia, 1942).

Simultaneous Inoculation of Rabies and Other Viruses

When mice are inoculated intracerebrally with a mixture of street virus and herpes, rabies disappears after a few passages, it being suggested that herpes causes too much nerve cell destruction for Negri bodies to develop (Levaditi, 1942 *a*)

Rabies virus is similarly suppressed when mixtures of louping ill and street virus are inoculated intracerebrally in mice (Levaditi, 1942 *b*) When rabies and Theiler's virus are inoculated together, the former disappears after a few passages (Levaditi and Noury, 1944), a similar result follows inoculation with neurotropic foot-and-mouth virus (Levaditi and Noury, 1943)

Using mixtures of street virus and LGI virus, Levaditi (1942 *c*) found that both survived at least 10 passages, probably as different cells are infected; both Miya-
[illegible] e brain.
[illegible] erent nerve cells, occurs between
[illegible] (Levaditi, 1943).

CHAPTER LXXVI

THE NEGRI BODY

ALTHOUGH the pathology of rabies had been carefully studied and various lesions

these structures (e g, 1905, and see 1909 *a*) It appears that at the same time, and independently, Bosc (1903 *c*) described similar structures in rabid dogs.

The significance of these bodies was soon affirmed from all parts of the world (see Abba and Bormans, 1905, Schiffmann, 1905, Negri-Luzzani, 1905; Bertarelli, 1905, Babès, 1907, 1915; Koch, 1910, Stimson, 1910, D'Amato and Faggella, 1910) A full review of the various papers that appeared on the question of Negri bodies within the first 10 years of the discovery was published by Negri-Luzzani (1913)

THE MORPHOLOGY AND OCCURRENCE OF THE NEGRI BODY

Morphology

Shape The body is usually rounded but may vary considerably. Thus Shortt (1935–6), who has made a special study, encountered the following shapes small spherical forms, larger spherical forms, oval forms, elongated forms, a form partly broken in two with a discrete form inside, triangular forms, bulging, ameboid, or budding forms

On microscopical examination there appears to be a definite homogeneous outer covering or capsule. Muratowa (1934), studying Negri bodies in mice, however, reported that the capsule was striated

Size. The Negri bodies vary from 0 25 μ to 20 μ or even 25 μ in the longest diameter. The smallest forms tend to occur in the early stages of the infection With the progress of the disease the bodies become larger, although small forms are still visible The largest forms of Negri body have been described in cattle, especially bullocks

Staining reaction The bodies are eosinophilic, although not always very strongly so, perhaps exhibiting a purple tinge Many bodies contain basophilic granules in their substance (*vide infra*). The bodies produced by *oulou fato* strains show a special tendency to basophilism The bodies are well stained by Mann's or Laidlaw's method A number of special stains and modifications has been introduced (Faraco, 1938, Parsons, 1939, Stovall and Black, 1940, McDonald, 1944).

Position. The bodies are found intracytoplasmically in nerve cells, they often occur between the nucleus and one of the corners of the cell Quite frequently they may be found in the prolongations of the cell, where they assume an oval shape

Internal structure From the taxonomical point of view the most interesting part of the Negri body is its internal morphology, for the larger bodies frequently contain one or more granules, nuclear-like in appearance These granules are variously known as *Innerkorperchen*, *Innerformationen*, nuclei, chromatin granules, chromatoid granules, or internal bodies or structures They have been studied by numerous workers (see, e g, Negri, 1905, 1909 *a*, *b*, Maresch, 1905, Williams and Lowden, 1906, Watson, 1913, Sanfelice, 1915, Covell and Danks, 1932, Muratowa, 1934, Shortt, 1935–6, Lépine and Sautter, 1946).

These granules are basophilic, and give a weak positive reaction to other tests

[1] References are appended at the conclusion of Ch LXXIX, p 887 et seq

for chromatin. It is possible that they do, in fact, contain true nuclear material. In the smaller types of Negri body there is usually only one granule, more or less centrally situated. In the larger forms there is usually a ring of several smaller granules around a larger central one. This central body may be compact, twisted, or somewhat diffuse in appearance. The granules are round or oval in shape, and usually measure from about 0.2 μ to 0.5 μ in diameter. Certain workers have described a condensation of cytoplasm round the granules resulting in an appearance as of a cluster of spores. Further, Watson (1913) described a rupture of the wall of the Negri body with an escape of spores to all parts of the nerve cell.

The term "Negri body," strictly speaking, should refer only to bodies containing internal structures. The term "lyssa body" should be used for eosinophilic inclusions that do not show basophilic granules In actual practice the term "Negri body" is usually used in a wider sense to include "lyssa bodies."

Negri Bodies in Man

Numerous authors have recorded the presence of Negri inclusion bodies in human material, although they are not so commonly present as in certain animals They have been found most commonly in the cerebrum, but also in the bulb and cord (see Acton and Harvey, 1911; Schiemann, 1912; Negri-Luzzani, 1913; Marie and Chatelin, 1919, Gerlach, 1925; Knutti, 1929; Dwijkoff and Bogoslowskij, 1929, Bassoe and Grinker, 1930; Schukrü-Aksel, 1934). Negri bodies occur in the endoneurocytes in the sensory fibers of the trigeminal and the sympathetic nerves of the face (Manouélian, 1936 *b*, *d*). Their presence has also been noted in the suprarenal (Marinesco, 1909). Busson (1930) reported that Negri bodies were absent in over 58 per cent. of those who developed rabies despite antirabies treatment.

Negri Bodies in Animals

Negri bodies have been found in a large number of animals, infected either naturally or experimentally with street virus, their presence has been reported in the following animals

Dog. The presence of Negri bodies in the hippocampus is the standard diagnostic test for the presence of canine rabies There has been some discussion of recent years as to whether the classical conception of the hippocampus as the site of election for the formation of Negri bodies is correct. This question was brought into prominence by the paper of Thomas and Jackson (1930), who found that the hippocampus did not appear to be the site of election in experimental infection of rabbits (see also Nicolau and Kopciowska, 1932 *a*). Muratowa (1934) came to the same conclusion by using mice. Naturally it was inquired if the same state of affairs might not apply to dogs, but the classical position has been defended by Horgan and McKinnon (1937). In diagnostic work, however, it is as well to examine material from various parts of the brain before giving a negative verdict In diagnostic work it is of value first to examine the cord, as the bodies may often be found there more readily (Greene and Breazeal, 1939–40) Negri bodies may be found in the endoneurocytes of the sensory and sympathetic fibers of the face.

Rabbit The bodies are readily demonstrable in the infected rabbit. Thus, after intracranial injection they occur most profusely in Ammon's horn, but also elsewhere in the central nervous system e g, in the cerebrum, *Purkinje cells*, cells of the basal nuclei, pons, spinal cord, and spinal ganglia. Thomas and Jackson (1930) stated that the bodies often occurred more frequently in the midbrain, especially [illegible] oculomotor nucleus than in the hippocampus The bodies may be found [illegible] fibers of the face, and in the nerve [illegible] Manouélian, 1935 *b*, 1936 *b*, *d*). After intrasciatic injection the bodies are found most profusely in the spinal ganglia and the cord, and only scantily elsewhere in the central nervous system (Negri-

Luzzani, 1913). Negri bodies may not be found in the brains of small rabbits as these tend to die more quickly (Proca and Bobes, 1940).

Guinea-pig. Negri bodies are very readily found in the infected guinea-pig (see Acton and Harvey, 1911). They vary in size up to about 16 μ, and may appear partly to encircle the nucleus.

Mouse. Muratowa (1934), in particular, studied the development of Negri bodies in mice. He stated that they appeared first in the midbrain around the central canal, and only later in Ammon's horn, they contained inner structures Certain workers have introduced a diagnostic test for rabies virus based on the demonstration of Negri bodies in infected mice (Webster and Dawson, 1934–5, Webster, 1936, 1937).

Some care would appear to be necessary in the study of Negri bodies in mice, as nonspecific structures have been reported (Nicolau, Kopciowska, and Balmus,

ventricle, Ammon's horn was practically free They also described another type of nonspecific inclusion which was larger (10–12 μ), and violet- or rose-colored, these bodies were found in the upper thalamic nuclei.

Levaditi (1944) found that Negri bodies appeared suddenly in Ammon's horn on the 5th day, and suggested that they developed from small oxyphil granules normally present in the nerve cell.

Cat. Negri bodies occur in rabid cats. They should not be confused with certain small granules (0.3 μ to 0.5 μ in diameter) which may be found in the nerve cell of normal animals (Negri-Luzzani, 1905, 1913). It appears that in the brains of cats infected with street virus the nerve cells containing the bodies may show degenerative change (Babès, 1915). This is unusual, as in most animals the cell containing the Negri body is more or less normal in appearance.

Bullock. Acton and Harvey (1911) reported that in this animal large forms up to 20 μ may be found Several may occur in one cell, and they are round or oval, possibly indenting the nucleus. The bodies have also been found in other bovines, generally assuming a large size.

Other animals in which Negri bodies may occur. Negri bodies have been found in a large number of animals infected naturally or experimentally with street virus-horse (Acton and Harvey, 1911), monkey (Acton and Harvey, 1911; Negri-Luzzani, 1913), fowl (Levaditi, Nicolau, and Schoen, 1926, Remlinger and Bailly, 1929 *a*), pigeon (Remlinger and Bailly, 1929 *c*), buzzard (Remlinger and Bailly, 1932 *a*), hedgehog (Sanfelice, 1915, Jonnesco, 1932 *a*), Chinese hamster (Yen, 1936 *a*), spermophiles (Jonnesco, 1932 *a*), capybara (Vianna, 1929), tortoise (Remlinger and Bailly, 1932 *a*), jackal (Acton and Harvey, 1911) and wolf, lion (Manouélian and Viala, 1922), goat (Acton and Harvey, 1911, Negri-Luzzani, 1913), pig (Negri-Luzzani, 1913), bat, mongoose.

Tissues in which Negri bodies may be found in animals.

Nervous system Negri bodies occur most commonly in the ganglion cells of the central nervous system, including the spinal ganglia They may also be found in the endoneurocytes of the sensory and sympathetic fibers of the face, and in the sympathetic ganglia (Manouélian, 1936 *b*, *d*), they may occur in bulbar nerves (Manouélian, 1936 *c*). They may, however, be found in various other parts of the body.

Salivary glands. Negri bodies may be found in the intraglandular ganglionic nerve cells in dogs, but not in the acini or the excretory canals (see Stefanescu, 1907, Manouélian, 1914) Bodies also occur in ganglion cells just beneath the lingual mucosa

Manouélian has stressed the importance of finding Negri bodies in the nerve cells of the salivary glands and mucosa of the tongue as affording the key to the

way in which the saliva becomes infected with virus (see, e.g., Manouélian and Viala, 1924, 1926, 1927, 1928 *b*; Manouélian, 1935 *a*, *b*).

Adrenal glands. After injection of virus into the adrenal glands very small Negri bodies were found locally (Levaditi and Schoen, 1935 *a*).

Cornea. The fact that Negri bodies may quite readily be demonstrated in the rabbit's cornea has only been pointed out comparatively recently by Levaditi and his coworkers Thus, Negri bodies appear in the corneal epithelium after inoculation of virus by scarification, or intra-ocular or intracerebral injection (Levaditi, Schoen, and Levaditi, 1934 *b*, *c*, Levaditi and Schoen, 1933, 1935 *a*, *c*, 1936 *a*). Negri bodies will develop in corneal cells undergoing regeneration after the application of croton oil (Levaditi and Schoen, 1936 *b*).

After intra-ocular injection small Negri bodies may be found in the retina (Levaditi, Schoen, and Levaditi, 1934 *a*).

In corneal infection the Negri bodies are usually small, measuring up to 4 μ, and are not very numerous in the initial stages, with the progress of infection they become more numerous. After corneal inoculation they appear contralaterally also. Corneal inoculation of guinea-pigs, cats, dogs, and monkeys does not produce Negri bodies, although they may be found occasionally in mice (Levaditi, Schoen, and Levaditi, 1934 *b*; Levaditi and Schoen, 1935 *a*).

Other organs. It appears that the cornea is the only epithelial tissue in which Negri bodies can develop. Thus, bodies were not found locally after injection of the rabbit's conjunctival, nasal, lingual, tracheobronchial, or intestinal mucosae, nor were Negri bodies found locally after injection of the parotid, testis, ovary, or skin (Levaditi, Schoen, and Levaditi, 1934 *c*, Levaditi and Schoen, 1935 *a*). It has, of course, already been mentioned that Manouélian found Negri bodies in the nerve cells beneath the lingual and buccal mucosae in animals.

Negri Bodies in Artificial Culture

Peragallo (1937) found Negri bodies in the chorio-allantois of the chick embryo They have been found in the brain and cords of chick embryos inoculated intracerebrally (Dawson, 1939) They do not seem to have been studied in tissue cultures prepared by the slide or Carrel techniques.

Negri Body Counts

An estimate of the extent of "parasitization" by Negri bodies can be obtained by counting 100 neurons and noting the number of bodies contained therein (see, e g, Nicolau and Kopciowska, 1933).

NEGRI BODIES PRODUCED BY *VIRUS FIXE*

It is generally held that *virus fixe* does not produce Negri bodies nearly so readily as does street virus, this property usually becoming lost after 25 to 45 passages (Schiffmann, 1905–6) However, Negri bodies may, in certain cases, be quite easily found. Thus, various authors have found Negri bodies in fixed virus infections of the rabbit (Manouélian, 1906, 1912, Ferré and Bonnard, 1908, Da Silva, 1927, Levaditi, Schoen, and Merger, 1932 *a*, Nicolau and Kopciowska, 1932 *a*, 1933, Lépine and Sautter, 1935) They may be found in infection with fixed *oulou fato* virus (Nicolau, Mathis, and Constantinesco, 1933), and Sassari virus (Lépine and Sautter, 1936) They can be found in animals infected with the highly active virus of *mal de caderas* (Remlinger and Bailly, 1931 *a*) These bodies are usually small (up to 7 μ), round or oval, and may possess an internal structure, although this is unusual. They may occur in Ammon's horn, but are sometimes said to be more profuse in the bulb (especially in the oculomotor nucleus) than elsewhere, this latter is important, as it may explain the failure of many workers to detect these bodies They may also occur in fixed virus infections of the following animals dog (Manouélian, 1912, Palawandow *et al.*, 1935), mice (Palawandow *et al.*), hedge-

hog (Palawandow *et al*), rat (Palawandow *et al*), guinea-pig (Palawandow *et al*), chameleon (Acton and Harvey, 1911).

Lentz bodies This is a convenient place to mention the bodies first described by Lentz (1909) and which go by his name. These structures measure about 10 μ in diameter; they occur extracellularly, being found between the ganglion cells in the central nervous system of rabbits injected with *virus fixe* They are eosinophilic and contain blue *Innerformationen* Similar bodies occur in "nervous distemper" of various animals.

NEGRIGENESIS OF DIFFERENT STRAINS OF VIRUS

There may be a certain amount of difference in the readiness with which various strains produce Negri bodies, that is to say, in their powers of negrigenesis (see Levaditi, Nicolau, and Schoen, 1926) Thus, working with the cornea Levaditi and Schoen (1935 *b*) found considerable variation between strains of street virus in this respect. It has been noted that the strains of street virus which do not readily produce Negri bodies are those which become fixed most quickly (*virus de rue renforcé*), and then do not produce inclusion bodies (Levaditi, Nicolau, and Schoen, 1924 *e*).

The Tunis strain of *virus fixe* was found to produce Negri bodies quite readily on intracerebral injection of rabbits, while the Tangier and Paris strains did not, ... 1935 *a, d*). ... the pro- ... passaged ... again to a certain extent their capacity for producing Negri bodies (Kopciowska, 1935 *a, b*, Nicolau and Kopciowska, 1935 *b*, 1936).

THE SPECIFICITY OF THE NEGRI BODY

There is world-wide unanimity on the specificity of the Negri body. That is to say, the occurrence of eosinophilic inclusions (usually with *Innerformationen*) in the nerve cells signifies the presence of a rabic infection. The appearance of the fully developed Negri body is unlikely to be mistaken for anything else With regard to the simpler lyssa body, however, various degenerative changes may simulate this homogeneous eosinophilic structure

There remain to be recorded some miscellaneous observations on specificity, none of these, however, offers any challenge to the trust rightly reposing in the specificity of the Negri body, being concerned mainly with structures that may be mistaken for lyssa bodies Thus, as mentioned above, lyssa-like bodies may be found normally in cats and mice and probably other animals as well Further, it was found that on rare occasions injections of Russell's viper venom produced lyssa-like bodies in the fascia dentata of guinea-pigs (Acton and Harvey, 1911). Also, rabbits injected with *B pyocyaneus* cultures might on occasions show lyssa-like bodies in their nerve cells (Acton and Harvey) Steinhardt *et al.* (1912) were able to produce small lyssa-like bodies by incuba- ... plasma Shortt and Lahiri (1934) produced ... by prolonged physiological stimulation of ... somewhat resembling Negri bodies may occur in the brain in canine distemper with encephalitis (Rifkin *et al*, 1945)

THE NATURE OF THE NEGRI BODY

ward much experimental data for the belief that normal cell constituents enter largely or entirely into the formation of the Negri body. The Negri body has been tested by almost all the usual methods, except, so far as we can discover, by micromanipulation The application of this technique (as used in other virus infections, see Ch X) should solve the problem of whether the body is purely degenerative or contains a living agent.

Experimental Observations

(*a*) *Microscopic.* The morphology of the body in ordinary stained preparations has already been sufficiently described. Darkground examination of unstained bodies shows that their structure is definitely granular and not homogeneous, the granules are not motile (see, e.g., Covell and Danks, 1932). Levaditi and Mezger (1933) studied Negri bodies (in mice) darkground, under a magnification of 1,800 diameters, and stated that the bodies (pansporoblasts) contained 5 or more cysts

(*b*) *Infra-red photography.* Guardrabassi (1935) examined Negri bodies in photographs taken by infra-red light, and found that they had a granular structure, even to the very periphery.

(*c*) *Vital staining.* Vital staining has been carried out by Covell and Danks (1932). According to these authors Negri bodies take up brilliant cresyl blue, Bremen blue, and Paris blue, the chromatoid granules and central mass appearing bluish-violet and vacuolated With Janus green B the chromatoid granules stain green and remain so after the color of the mitochondria has faded.

(*d*) *Tests for the presence of normal cell constituents*· (1) *Chromatin.* Negri bodies, as a whole, give a negative Feulgen reaction (Paul and Schweinburg, 1926), although a faint positive test many be given by the chromatoid granules (Covell and Danks, 1932). Similar results are obtained by Millon's and MacCallum's tests (*Covell and Danks*). *This finding suggests that only the chromatoid granules contain* any nuclear chromatin (see also Lépine and Sautter, 1946).

(2) *Golgi apparatus and mitochondria.* Negri bodies do not react to stains for the Golgi apparatus (Fañanás, 1922, Levaditi, Schoen, and Mezger, 1932 *b*), nor do they react to stains for mitochondria (Schiffmann, 1905–6; Levaditi, Schoen, and Mezger, 1932 *b*). Here one may mention [illegible] *been* found in the ganglion cells in rabies (e.g., [illegible] bably of mitochondrial origin and not lyssa bo[illegible] e cell in which Negri bodies are found may show degenerative changes in the mitochondria and Golgi apparatus, but these changes are more likely to be the result than the cause of the inclusion (Covell and Danks, 1932)

(3) *Neurofibrillar network.* Goodpasture (1925) found that a degenerative change in the neurofibrillar apparatus of the ganglion cells might result in the production of bodies identical with lyssa bodies.

(4) *The Nissl substance and nuclear chromatin* Covell and Danks (1932) believed that the virus produces chromatolysis, with an accompanying alteration in the Nissl substance (see also Mackenzie, 1897, Cowdry, 1928). They suggest that iron-containing basophilic nuclear material is extruded into the cytoplasm where it is eventually converted into the oxyphil matrix of the Negri body In the center basophilic material remains unaltered as the *Innerformationen*

Another line of approach to the problem of the Negri body has been to *infect* animals with virus and to kill them at varying periods thereafter, and study the Nissl substance Nicolau and Kopciowska (1934 *a*) injected rabbits intrasciatically with street virus They found that Negri bodies appeared in the neurons of the corresponding ganglia from the 7th day onward. During these 7 days of incubation they found various changes

(*a*) The Nissl bodies agglutinated, eventually to form chromophil masses of irregular appearance.

(*b*) From these masses a number of regular basophilic spherical bodies was formed

(*c*) Next a small red dot appeared in the center of these bodies, the redness spread, and eventually small Negri (lyssa) bodies were formed Oxyphil material was further laid down and these smaller bodies might fuse to form a larger.

(*d*) The cells with the best-formed Negri bodies contained no Nissl bodies. [illegible] - those cells normally richest [illegible] idies, e g, cells in the spinal

From this work they concluded that the Negri body was the expression of the defence of the cell. A successful defence meant preservation of the cell with a Negri body contained therein Unsuccessful defence meant possible destruction, without the presence of any Negri body.

(*e*) *Micro-incineration* Micro-incineration studies were carried out by Covell and Danks (1932), [illegible] injection with street [illegible] white in color, wh [illegible] iron. There was no evidence for the presence of a membrane, nor for the presence of individual parasites in the body. If the internal structures were nuclear, ash-free areas would have been expected, but none such occurred In the cells containing [illegible]

Theoretical Considerations

(*a*) *The Negri body as a protozoon* It was only natural that in the early days, workers should believe the Negri bodies to be protozoal organisms (see Negri, 1903 *a*, *b*, 1909 *a*, *b*, Di Vestea, 1904, Volpino, 1905–6, Williams and Lowden, 1906, Calkins, 1909) To them the appearance of the internal structures was very suggestive of a nucleus, and the bodies appeared to be of the same nature as malarial or other protozoal organisms Calkins gave the name *Neurocytes hydrophobiae* to the rabies organism

More recently the protozoal theory has been upheld by Levaditi and his co- [illegible]

are known as spores, these pass along the nerves from the site of the wound and reach the central nervous system. Second, arrived at the central nervous system they undergo an intracellular proliferation to form the pansporoblast or Negri body The pansporoblast consists of an envelope formed from the cytoplasm of the nerve cell, and contains a number of spores, each with chromatin material. The development of this pansporoblast depends on the virus and on the host. Thus, if the virus is virulent it may destroy the nerve cell before a pansporoblast can form For instance, in *virus fixe* infection the virus is said to be of such virulence that these pansporoblasts are not usually found If *virus fixe* is injected into a rabbit already infected with street virus, then Negri bodies occur only rarely. *Virus fixe* seems to be able to prevent the pansporoblast stage from occurring.

Levaditi believes that the Negri body is one stage of a protozoon analogous to *Encephalitozoon cuniculi*, or *Glugea lophii*, both of which protozoa occur in brain tissue He has given the name *Glugea lyssae* to the rabies organism. Only one phase, that of the Negri body (pansporoblast), is visible, in the other phase (spores) the organism is invisible and filtrable.

Of somewhat similar implication is the term *Encephalitozoon rabiei* applied to the rabies organism by Manouélian and Viala (1924). These workers have described small fusiform or pyriform bodies measuring 1 μ to 2 μ in diameter, which are said to contain chromatin granules and to be surrounded by a membrane. Variable numbers of bodies may occur inside cells, and they may fuse to form the larger Negri body. The bodies are held to represent a spore-like phase of a protozoon.

Veeraraghavan (1944) has found what he takes to be a protozoon in the nerve cells in the midbrain in guinea-pigs, also in dogs and jackals. Various forms of the parasite are described and it appears to be filtrable. It is possible, he holds, that the Negri body is a stage in the life cycle of this parasite.

Levaditi's theory is certainly most attractive and ingenious. In particular it obviates the necessity for always finding Negri bodies in sites where the virus can be shown to exist; because, under certain circumstances, only the filtrable phase is present, he suggests; the pansporoblast phase is only facultative—it need not always occur.

It is difficult, however, to see how this theory can be regarded as anything but unproved. Although there is little or no evidence actually against the theory, there is none that proves that the rabies virus is a protozoon. The micro-incineration studies of Covell and Danks (1932) certainly do not support the protozoal theory, they were unable to find any evidence of a definite cyst wall or membrane to the Negri body, or of a protozoal nucleus. The delegates attending the First International Conference in Paris in 1927, after examining preparations by Levaditi and Manouélian, were unable to decide whether the Negri body represented a living organism or was merely of degenerative nature. During the past 10 years, the observations that have been brought forward have tended more to emphasize the rôle of normal cell constituents in the formation of the Negri body.

(*b*) *The Negri body as a colony of virus particles.* The Guarnieri body of variola-vaccinia, the Bollinger body of fowl-pox, and the inclusion body of molluscum contagiosum all consist of a mass of elementary bodies held together as if in a colony. The question naturally arises has the Negri body the same type of structure?

There is certainly evidence that the Negri body contains granules, and even in the early days it was suggested that it was a chlamydozoon containing elementary bodies in a sticky matrix (Volpino, 1905–6, Prowazek, 1907, Babès, 1907, Lipschutz, 1919). More recently the granularity of the Negri body has been confirmed by darkground examination and infra-red photography. Some difficulty arises in con-
nec
boc
virt
of

(*c*) *The Negri body as a product of the cell.* It has, of course, been suggested by histologists from the earliest days that the Negri body is a purely degenerative structure formed from the cell constituents. That the degeneration must be of rather a particular and specific type was admitted, for the nerve cell containing the body usually appears comparatively little damaged. Thus, it has been suggested that the Negri body is derived from the following structures.

1. *The nucleolus.* This theory was advanced by Acton and Harvey (1911, 1912–13), who regarded Negri bodies as the result of an interaction between extruded nucleoli and the cytoplasm. They found that the nucleoli in the nerve cells of rabid animals stained abnormally, and were often fragmented. These fragments, they suggested, were discharged into the cytoplasm to constitute the iron-reacting granules in the Negri bodies. This theory is somewhat similar to that expressed more recently by Covell and Danks, and already alluded to, in which they suggest that nuclear chromatin is extruded and undergoes oxyphil degeneration.

2. *Neurofibrils.* It has already been mentioned that Goodpasture (1925) found

that a degenerative change occurred in the neurofibrillary network of the ganglion cells. This change produced eosinophilic Negri-like bodies without inner structures This theory would serve to explain the formation of lyssa bodies but not of true Negri bodies

(3) *Nissl substance and nuclear chromatin.* As mentioned, a number of workers has stressed the rôle of the Nissl substance. It appears that lyssa-like bodies may develop by oxyphil degeneration of this material. Others suggest that basophil

because if the oxyphil change begins at the periphery and spreads inwards, one would reasonably expect the center of some of the larger bodies to be still basophil and unaffected by the change. This theory is the only one of this type that can reasonably explain the *Innerformationen* (Black, 1940).

Conclusions

manipulation, and to inject such a structure into an animal It is possible that electron microscopy may prove of value.

CHAPTER LXXVII

IMMUNITY MECHANISMS IN EXPERIMENTAL RABIES

A. NATURAL IMMUNITY

A NUMBER of species of animals is quite insusceptible to infection with rabies virus E , an occasional resistant animal n 38 *b*; Jonnesco, 1934 *a*, 1936 *a*, J whether the serum of animals naturally resistant to rabies is rabicidal. Thus Kondo (1922) reported that the sera of two naturally refractory dogs were rabicidal. Phisalix (1926) found the serum of the adult hedgehog to be rabicidal, but Jonnesco (1927 *a*) could not confirm this point. The serum of vipers and eels was found to be rabicidal (Remlinger and Bailly, 1928 *a*).

It is not clear whether the offspring of immune mothers are immune or not Remlinger (1908 *c*) claimed that hereditary immunity does occur, but Babès and Talasescu (1894) could not demonstrate any such resistance.

B. ACTIVE ACQUIRED IMMUNITY

Animals become resistant to rabies on recovery from infection with street virus, but this is a rare event, and of little practical importance. A considerable amount of work has been carried out on the development of active immunity by vaccination, prior to infection ("challenge") with a test inoculum of live virus.

Species of Animal Immunized and Route of Injection

Of the laboratory animals, dogs, rabbits, monkeys, guinea-pigs, rats, and mice have all been rendered refractory by vaccination with live or chemical vaccines to subsequent challenge doses of living virus. Many of the early workers claimed to have demonstrated this acquisition of immunity, although it must be said that their experiments were not always well controlled, the state of immunity was not always of a high order and could usually be broken down by a sufficiently large challenge dose, especially given intracerebrally or intra-ocularly (Pasteur *et al*, 1884, Galtier, 1881, Bardach, 1887 *a*, Roux and Nocard, 1888, Roux, 1888 *b*, Protopopoff, 1888).

Larger animals such as goats, sheep, and horses have also been immunized, mainly with a view to producing antirabies serum

The important question of prophylactic immunization of dogs is discussed in a separate section below

Immunizing injections can be given subcutaneously, intravenously, intraperitoneally (Remlinger, 1908 *b*, Lieou, 1940), percutaneously (Remlinger and Bailly, 1927 *d*, 1928 *a*, de Georges, 1926), or rectally (Remlinger, 1907 *b*) Some have claimed that immunizing injections can be given intracranially (Phisalix, 1926, Tzekhnovitzer and Goldenberg, 1930), but others disagree (Remlinger and Bailly, 1928 *c*, Loeffler and Schweinburg, 1930–1) Intraspinal injections of irradiated virus were found to immunize rabbits (Kasahara and Sha-Shi-Nan, 1940)

Source of Vaccine

Numerous preparations have been used to provoke active immunity.

(*a*) *Living*, or attenuated, *virus fixe* can be injected intraperitoneally, sub- or intra-cutaneously (see, e g, Hoegyes, 1887, 1889 *a*, *b*, *c*, Fermi, 1908, Stuart and

[1] References are appended at the conclusion of Ch LXXIX, p 887 *et seq*

Krikorian, 1932 *a*, Shortt *et al*, 1934–5, Webster, 1939 *b*, Casals and Webster, 1940). Owing to the risk of death, and the difficulty of giving large quantities, this method is not widely used, although resistance can be stimulated by live virus, especially against a peripheral challenge.

Webster (1939 *b*) found that mice were immunized with the Hoegyes and Harris live vaccines within 10 days to withstand 100 intracerebral lethal doses, and that this resistance persisted for at least 9 months. In experiments with dogs Shortt *et al* (1934–5) found that the most effective method of protection was to give live virus after a course of carbolized vaccine.

(*b*) *Killed vaccines* of various types have been used. Certain earlier workers did not carry out adequate controls and others probably used relatively inert material Provided, however, that the vaccine contains a sufficient quantity of virus, and that the strain is fully antigenic, a satisfactory state of immunity can be induced in dogs, monkeys, rabbits, guinea-pigs, and mice by phenolized, etherized, chloroformized, and formolized vaccines, there is evidence that the chloroform-treated vaccines are somewhat superior (see, e g, Remlinger, 1919 *c*, Gloster *et al*, 1929–30, Kelser, 1930, Tzekhnovitzer and Goldenberg, 1930, Stuart and Krikorian, 1932 *a*, Okuwada, 1933, Shortt *et al*, 1934, 1937, Jacotot, 1938, van Stockum, 1938, 1941; Boecker, 1938–9, Lépine and Sautter, 1939, Webster, 1939 *b*, Johnson and Leach, 1940 *b*, 1942, Leach and Johnson, 1940 *b*, *c*, 1942, Webster and Casals, 1941, 1942 *a*, Veeraraghavan, 1946 *b*).

Alumina gel may be added to formolized virus (Jacotot, 1947).

Guinea-pigs have been immunized to withstand intramuscular injection of test virus by 2 injections of brain inactivated with mustard (Tenbroeck and Herriott, 1946).

Injections are usually given repeatedly, as large doses are needed, and by the subcutaneous or preferably the intraperitoneal route. Resistance is usually sufficient to withstand an intracranial test dose. It should be realized that the doses used have usually been very large, monkeys for example receiving doses similar to those given to human beings Webster (1939 *b*) found that a phenolized and a chloroformized vaccine had to be used in mice in a dose approximating 5 times that advocated per gm of body weight for man

(*c*) Dogs, rabbits, guinea-pigs and sheep have been immunized by *mixtures of virus and antiserum*, neutral or otherwise (Marie, 1902; Remlinger, 1904 *f*, 1906 *e*) Rabbits have been immunized by neutral mixtures of hedgehog (naturally immune) serum and *virus fixe* (Phisalix, 1926) Shortt *et al* (1934–5), however, did not obtain definite evidence of the value of serum combined with virus

(*d*) Virus grown in *tissue cultures* can be used to immunize animals either in its live state, formolized, or when inactivated by ultraviolet light (Hodes *et al*, 1937, 1940, Webster, 1937, 1938, Kligler and Bernkopf, 1938 *a*, 1941 *b*, Webster and Casals, 1941) Webster (1938) prepared the vaccine as follows 4 c c of 10 per cent monkey serum in Tyrode + 0 02 c c of minced mouse embryo brain is inoculated with 1 c c of a 1 100 dilution of infected mouse brain, the cultures are incubated at 37° C for 3–4 days in a 50 c c Erlenmeyer flask, passage is performed with the supernatant obtained after centrifugation. Veeraraghavan (1946 *b*) claimed that his "cell-free" culture virus immunized guinea-pigs against fixed virus injected subdurally, and rabbits against street virus injected in the neck

(*e*) The principle of *inactivation by exposure to U-V light* can also be extended to infected brain tissue (Webster and Casals, 1940 *a*, 1941, 1942 *a*, *b*, Levinson *et al*, 1944) Such vaccines are considerably more potent than tissue cultures or chloroformized and phenolized vaccines, but must not be irradiated for too long, or antigenicity is decreased They appear to contain inactivated virus The method adopted by Webster and Casals (1942 *a*) was as follows Two-month old beagle dogs were inoculated intracerebrally with infected mouse brain and when prostrate their brains were removed, weighed, triturated and diluted with buffered distilled

water to make a 5 per cent suspension, which was centrifuged. To be effective this supernatant had to titer 330,000 or more mouse intracerebral doses per c.c. The supernatant was rendered avirulent by exposure to U-V light for 35 minutes. Intraperitoneal injection of this vaccine would protect mice against 10,000 MLD (as compared with a chloroformized vaccine—1000 MLD). Of 35 dogs given 30–40 c.c. of this irradiated vaccine in one dose, all were protected save one, and when the vaccine was concentrated sixfold all of 24 animals were protected. Mortality in the unvaccinated control dogs was 84 per cent. Habel (1947), using the Levinson type of lamp, has produced a vaccine considerably more potent than that used by Webster. Later he introduced an alternative lamp (see Habel and Sockrider, 1947)

(*f*) *Virus inactivated by methylene blue* is still antigenic (Galloway, 1934), but is unlikely to be sufficiently active for use in antirabies treatment (Shortt and Brooks, 1934–5). It is probable that such vaccines are ineffective unless some live virus remains.

The Effect of Age on Development of Immunity

W-Swiss mice of 60 or more days old were more readily immunized than 20-day or younger mice; thus, 1600 doses injected peripherally immunized 60-day-old mice against 100 or more doses given intracerebrally, but 20-day mice against only 10 or less doses (Casals, 1940 *b*, Casals and Webster, 1940).

Technical Methods in Testing the Degree of Immunity

It has been usual to challenge immunized animals after their course of vaccine by injection of street or fixed virus intracerebrally, intra-ocularly, subcutaneously, or intramuscularly. Various workers, using less effective vaccines, have experienced difficulty in immunizing animals sufficiently to withstand intracerebral or intra-ocular injections of test virus. It was generally held that the intracranial test was too severe. Newer and more potent vaccines may, however, immunize sufficiently for an intracranial test dose to be withstood. It has been found considerably easier, however, to protect against a peripheral injection of test virus (see Webster, 1939 *a*, 1942).

In tests for the immunizing potency of vaccines it is always desirable to inject the test dose of virus in such a way that as nearly as possible 100 per cent. of the control unvaccinated animals develop rabies Unless injected intracerebrally or intra-ocularly, however, it is very difficult to secure infection of more than 50–75 per cent of controls. It is also desirable to use an accurately titrated amount of test virus, and for this purpose it is now usual to employ mice. Albino rats were recommended by Cornwall and Beer (1925–6 *c*). Various methods have been introduced in recent years for assaying the immunizing potency of antirabies vaccines.

1. Webster's mouse immunity test

Webster (1939 *b*, 1940, 1941) introduced a simple test carried out as follows

Dilute the vaccine 1/10 and use 1/8th of the stated dose (i e, 1/80th) Set aside 16 3-week-old mice for the test and a similar number as controls In the case of canine vaccines, 1 dose is given intraperitoneally to each test mouse When testing human vaccines, the 16 mice are injected intraperitoneally for 3–6 days Three weeks after the first injection of vaccine, the 16 test mice are divided into 4 lots and tested intracerebrally with 1, 10, 100, and 1000 MLD. The controls are treated likewise Sometimes the challenge dose is given intramuscularly in the gastrocnemius muscle, using from 2–32 MLD in doubling dilutions This test is more sensitive and detects relatively weak immunizing capacity. The test and control mice are examined for 60 days

Webster regarded the mouse immunity test as of considerable value in indicating the immunizing efficiency of antirabies vaccines Remlinger and Bailly (1943 *c*, *d*), on the other hand, question whether mice are really suitable for such tests, and suggest that they are unduly susceptible to rabic infection, virus probably not spreading to the nervous system by means of nerve paths, as occurs in man and larger laboratory animals

2. *Wyckoff's method*

The following method has been used by Wyckoff (1940-1, Wyckoff and Beck, 1940, Wyckoff and Tesar, 1941). Ten 3-week-old mice are vaccinated daily for 6 days with 0 6 c c of a 1/9 dilution of vaccine given intraperitoneally. Six days later, 5 mice are injected intracerebrally with 100 MLD, and 5 mice with 1000 MLD of homologous fixed virus The result is assessed 2 weeks later

3 *Habel's method.*

Habel (1940 a) uses 30 Swiss mice, all of one sex (preferably female), and administers 0 25 c c of vaccine diluted to contain 0 5 per cent brain tissue (i e, usually 1/10) intraperitoneally every 2nd day, giving 6 doses in all, 18 controls are needed Fourteen days from the first dose of vaccine, the challenge dose is given to the immunized animals The test virus consists of fresh passage brain tissue, and is titrated by dividing the controls into 3 groups and injecting 0 03 c c. of 10^{-5} to 10^{-7} dilution intracerebrally. The immunized mice are divided into 5 groups, and injected intracerebrally with 0 03 c c. of 10^{-1} to 10^{-5} dilutions The mice are observed for 21 days The MLD is the highest dilution causing death from rabies in at least 3/6 controls The end-point of the immunity in the vaccinated mice is the lowest dilution of the test virus which causes at least 3/6 to survive The number of MLD protected against can be determined easily by the number of times the dilution giving the end-point in vaccinated mice is more concentrated than in the controls When the end-point is irregular, the 50 per cent end-point should be calculated by Reed and Muench's (1938) method

This technique is applicable to all types of killed virus vaccine When testing live or attenuated viruses the 6 intraperitoneal doses must be graded Habel considered that the minimum requirement of an efficient vaccine was that it should protect against at least 1000 MLD Habel and Wright (1948) have recently described improvements in this method of assay, designed to give more consistent results

4. *Methods using dogs*

Dogs have been extensively used in this type of work. For example, they have been challenged after 4 weeks with street virus injected in the masseters, under these conditions only 50-60 per cent of unvaccinated controls die of rabies (Johnson and Leach, 1940 *b*, 1942, Leach and Johnson, 1940 *b*, *c*, 1942)

Webster and Casals (1940 *b*) have recommended the following test Beagle dogs, 4-6 months old, are immunized with the vaccine, as required Three weeks later they are challenged by an injection into the neck muscles on either side of 0 25 c c of varying dilutions of a passage strain, 3 groups of animals receiving 1, 10, or 100 lethal doses Suitable numbers of controls are similarly injected The test and control dogs are observed for 2 to 5 months

Miscellaneous Observations

The effect of alcohol on development of immunity It has been stated that alcohol-treated animals do not acquire immunity (Deléarde, 1897) Remlinger and Bailly (1932 *a*), however, found no difference in the resistance of normal and alcohol-treated groups

Nonspecific results of vaccination These results were summarized by Cruveilhier, Nicolau, and Kopciowska (1935, see also 1931, and Nicolau, Cruveilhier, and Kopciowska, 1932)

1 Treatment raised the *Bact typhosum* agglutinin titer of a rabbit previously immunized against this organism
2 The hemolytic power of the serum was likewise increased in rabbits previously injected with sheep's red cells
3 The general health of the animal was improved
4 Antirabies treatment applied to lactating rabbits carrying a Shope's tumor caused it to disappear (Haguenau, Cruveilhier, and Viala, 1937)

There has been some discussion whether long courses of antirabies treatment render animals more susceptible to subsequent injections of virus Loeffler and

Schweinburg (1930) claimed that this was so, but Remlinger has disagreed (Remlinger *et al.*, 1931 *b*, Remlinger and Bailly, 1931 *a*)

Histological reaction in vaccinated animals. Immunization by the desiccated cord method was found to produce definite lesions in the central nervous system (Nicolau, Cruveilhier, and Kopciowska, 1931 *a*, *b*; Cruveilhier, Nicolau, and Kopciowska, 1935). These reactions have been attributed to a mild infection, the virus is only attenuated and probably the reaction is one of defence. Some cellular infiltration may be found in the meninges In the cerebral cortex there are areas of mononuclear infiltration and microglial reaction. Very marked satellitosis of nerve cells occurs in the mesencephalon and diencephalon. In the cord, the posterior horn is infiltrated with mononuclear cells, satellitosis may be seen. The Nissl bodies may be degerate and agglomerated in large masses. In the spinal ganglia a parenchymatous infiltration of mononuclears and a proliferation of capsular cells are usually present. Lépine and Sautter (1938) have reported that nuclear inclusions (see p. 831) develop in the brain after injection of phenolized virus; this they attribute to the presence of living virus in their vaccine.

The reticulo-endothelial system in acquired immunity. Immunity can be acquired by splenectomized dogs (Plantureux, 1932). A previously acquired immunity can, however, be abolished by blockade with India ink (Loeffler and Schweinburg, 1933–4). Further, Loeffler and Schweinburg (1932 *a*) have shown that the serum of animals blockaded with India ink and vaccinated is less virucidal than that of unblockaded animals, in addition, these animals are less protected against reinfection than unblockaded animals. The reticulo-endothelial system would seem, therefore, to play a definite rôle in resistance to infection

C. PROPHYLACTIC ANTIRABIES TREATMENT OF ANIMALS

In various parts of the world where rabies is endemic, prophylactic immunization of dogs and sometimes other animals has been carried out, in an attempt to limit the spread of the disease (Remlinger, 1919 *a*, Plantureux, 1926 *a*, Vallée, 1927, Remlinger and Bailly, 1932 *a*, 1934 *c*; Balozet, 1938 *a*, *b*, Gautier, 1940) The following vaccines have been used

(*a*) Living *virus fixe* vaccines (Roux and Nocard, 1888, Galtier, 1897, Schnurer, 1905, Remlinger, 1921, Jonesco, 1930 *b*)
(*b*) Hoegyes' vaccine (Aujzesky, 1922)
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(*f*) Miessner's (1912) vaccine
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More use has, however, been made of the following methods

(*h*) Etherized vaccines (Remlinger, 1918 *a*, 1919 *a*, 1925 *c*, Bailly, 1926, Remlinger and Bailly, 1930 *f*, 1932 *a*)
(*i*) Chloroformized vaccines (Kelser, 1930, Schoening, 1930, Barnes *et al*, 1934; Webster, 1939 *b*, 1940, Leach and Johnson, 1940 *c*, Webster and Casals, 1942 *a*)
(*j*) Formolized vaccines (Konieff and Ramsine, 1928, Plantureux, 1935, 1938)
(*k*) Phenolized vaccines, attenuated or killed (Umeno and Doi, 1921, Kondo, 1922, Kitt, 1925, Velu *et al*, 1926, 1928, Plantureux, 1926 *a*, *d*, Legezynski and Markowski, 1928 *a*, Markowski and Legezynski, 1928 *a*, Dos Santos, 1929, Schoening, 1925, 1928, 1930, 1931)
(*l*) Irradiated vaccines are highly effective immunizing agents (see p 845), Webster and Casals, 1942 *a*).

Vaccines are usually given in one dose subcutaneously or preferably intraperitoneally. It is very questionable how much resistance has been induced by many of

the above mentioned workers, for it appears that a considerable number of vaccines available commercially has been possessed of only very slight immunizing activity (Johnson and Leach, 1940 *b*, L. T. Webster, 1939 *b*, 1940, L. T. Webster and Casals, 1940 *b*, 1942 *a*, W. J. Webster *et al*, 1940; Wyckoff and Beck, 1940, Wyckoff and Tesar, 1941). Thus L. T. Webster (1939 *b*, 1940) tested a considerable number of phenolized preparations, and found that none immunized mice against a subsequent intracerebral challenge dose of 1 MLD; chloroformized vaccines, however, im- [illegible] -se recommended, proportionately, [illegible] phenolized vaccines were found [illegible] vaccines produced a significant degree of resistance, especially if given in 10 c c. amounts intraperitoneally (see also Webster and Casals, 1940 *b*, 1942 *a*, Leach and Johnson, 1940 *c*).

There is considerable doubt about the effect of mass vaccination of dogs on the incidence of rabies, and it would appear from what has been said above, that much of the vaccination work done in the past must have been completely ineffective. Provided a potent chloroformized or newer irradiated type of vaccine is used, in doses proved experimentally to be antigenic, it would seem reasonable to expect some decrease in the incidence of rabies in countries with large dog populations

Johnson in Alabama has found that a significant degree of immunity can be conferred on dogs against an intramuscular challenge of street virus one year later; a single subcutaneous injection of 5 c c. of potent vaccine was effective, but 3 weekly doses induced a greater degree of resistance (see *Public Health Reports*, 1947), within recent years, on the basis of this work, several American organizations have recommended the annual prophylactic vaccination of dogs as an effective means of controlling canine rabies.

D. ANTIRABIES TREATMENT OF EXPERIMENTAL ANIMALS AFTER INOCULATION WITH LIVING VIRUS

The most obvious method of assessing the efficiency of antirabies vaccines for use in the treatment of human beings is to test their effect in preventing rabies when administered to animals after experimental infection This method simulates as closely as possible what happens when vaccines are used in practice. A number of experiments has been carried out in the past, and the question has been critically reviewed by Webster (1939 *a*, 1942) whose papers have attracted the widest interest Numerous workers have obtained negative or virtually negative results. Thus, following intracranial injection of street or fixed virus in rabbits, dogs, and rats, courses of attenuated, diluted, and phenolized vaccines have failed to prevent the onset of rabic symptoms in a significant proportion of animals (von Frisch, 1887, Hoegyes, 1889 *a*, *b*, Fermi, 1908).

Workers in India infected rabbits and monkeys by corneal scarification, and treated them with phenolized, etherized, and phenol-ether vaccines The rabbits were not protected from rabies, about 60 per cent of vaccinated monkeys died, as compared with 72 per cent. of controls (Cunningham and Malone, 1930, Cunningham *et al.*, 1933) This work in India was continued with monkeys, and in a series of experiments well over 1000 animals were used Infection was usually secured by injecting street virus intramuscularly in the neck, phenolized vaccines with and without antirabic serum being administered subsequent to infection Although some protection was achieved, the results were most disappointing, as it was not possible to protect as many as 50 per cent. of animals It should be remembered that in experiments of this kind, approximately one quarter of the control animals fail to develop rabies, as street virus does not infect regularly by the intramuscular route (Shortt *et al*, 1934, 1934–5, Covell *et al*, 1936–7)

Other workers have carried out similar experiments, and although some vaccines may have appeared to protect, the numbers of animals used have been too small to draw conclusions (Remlinger, 1919 *c*, Lépine and Sautter, 1937)

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Virus strains vary considerably in their capacity to stimulate the production of antibodies, some scarcely doing so at all (Remlinger and Bailly, 1931 *b*). It has been noted that there is little correlation between the antirabic titer of the serum and the quantity of inoculum, and that the titer may fluctuate, especially in sheep (see Remlinger, 1907 *d*, Marie, 1927 *b*). A strongly antirabic sheep serum in a quantity of 1 c c. may neutralize 40 times its bulk of 1 per cent. virus suspension (see Marie, 1907 *a*). Habel (1940 *b*) produced a hyperimmune rabbit serum by repeated doses of phenolized vaccine followed by inoculation of live virus Later, he found that intracutaneous inoculation of live virus was more effective (1945) The rabicidal properties may persist for a considerable time after inoculation (Webster, 1936)

Nature of the rabicidal antibody. A number of authors has investigated the nature of the rabicidal antibody, and the rabies antigen-antibody reaction (Marie, 1905 *a*, 1908, 1912 *a*, Kondo, 1922; Nikolajewa, 1925, Lubinski and Prausnitz, 1926, Isabolinski and Zeitlina, 1929, Shortt *et al.*, 1934–5). There is some difference of opinion with regard to the nature of the reaction between antigen and antibody, some saying it occurs in multiple propertions, some saying the union is stable, and others saying it is easily broken down Kondo states that the optimum time and temperature for union between antigen and antibody is 2 hours at 37° C., or 20 hours at 18° C to 20° C (in the dark). Friedemann *et al* (1944) found that virus and antibody combined *in vitro* even if the immune serum was highly diluted The properties of the rabicidal antibody are as follows It is destroyed by 80° C. for half an hour, but there is no change at temperatures below 70° C applied for half an hour It is associated with the globulin fraction (Shortt *et al*) Immune body can be absorbed by virus.

Antirabic properties of immune tissues Kubes and Gallia (1944) have claimed that the brain tissues of mice immunized with antirabies vaccines contain substances neutralizing the virus *in vitro*, and the more the animal has been immunized the greater this neutralizing effect They state that the results of the tissue neutralization test agree with those obtained by protection experiments

Virus was found to disappear from the site of injection more rapidly in immunized mice and guinea-pigs than in controls, and failed to invade the nervous system (Habel, 1941 *b*, Kligler and Bernkopf, 1943)

The significance of the rabicidal immune body As Stuart and Krikorian (1932 *a*) emphasized, there is divergence of opinion as to whether rabicidal substances indicate resistance to infection or not They could not detect any mathematical relationship between rabicidal content of the serum and the degree of resistance to infection Jonnesco (1932 *b*) also failed to elicit any parallelism between rabicidal power of the serum and resistance to reinfection (see also Habel, 1941 *b*, Kubes and Gallia, 1944) Further, as Remlinger and Bailly (1928 *a*) point out, in the human being rabicidal powers do not necessarily develop after vaccination, although the patient appears to be protected against rabies Moreover, fatal cases of rabies have been known to have a highly rabicidal serum In vaccinated mice, although immune bodies and resistance usually develop together, there may be lack of correlation (Webster, 1936, Casals, 1940 *b*) Fowls may be rendered resistant to virus yet show no antibodies (Kraus and Maresch, 1902) On the other hand, it has been said that in rabbits, those showing rabicidal properties are always immune to reinfection (Cruveilhier and Viala, 1937)

F. PASSIVE IMMUNITY

A number of workers has tested the effect of antirabic serum on experimental rabies Thus, they have been able to prevent the development of rabies in experimental animals by injections of serum made just before or just after that of the virus (e g, Babès and Lepp, 1889, Babès and Talasescu, 1894, Kondo, 1922, Pono-

Fermi (1908) obtained more satisfactory results by infecting rats subcutaneously with *virus fixe*, and treating with phenolized vaccines. Large doses of vaccine were, however, needed (over 20 c.c. per rat) to protect the infected animals (see also Mulas, 1936, who worked with rabbits).

Habel (1940 *a*), working with guinea-pigs injected intramuscularly with street virus, was able to protect 16/27 by a series of 21 daily injections of 0.5 c.c. phenolized vaccine; 4/5 controls developed rabies.

In another series of experiments, mice infected intramuscularly with street virus were not protected by subsequent injections of phenolized virus, although chloroformized vaccines occasionally protected, when, however, 5 doses of an irradiated vaccine were given, rabies was prevented in most cases (Webster, 1939 *b*, Webster and Casals, 1942 *a*). Habel (1947), using irradiated virus, demonstrated some protection against death from street virus in guinea-pigs immunized after infection.

We may conclude, therefore, that it is difficult to protect animals by antirabies treatment given subsequently to infection. Large doses of phenolized, chloroformized, and potent irradiated vaccines may, however, secure protection in the smaller laboratory animals.

E. ANTIBODY PHENOMENA IN EXPERIMENTAL RABIES

The precipitating antibody. According to a majority of authors, precipitating antibodies do not develop (Burmeister, 1915, Kondon, 1922; Lässer, 1927, Schultz, Bullock, and Brewer, 1928). Of more recent years, however, Havens and Mayfield (1932 *a*, *b*, 1933) have claimed that flocculation occurs between rabies virus and antiserum. Further, they find that antigen can absorb its specific immune body. They have used this method for investigating the antigenic structure of rabies virus.

The complement fixing antibody. Rabbit, horse, or other animal immune serum may fix complement with salivary gland extract from rabid animals or human beings (Nedrigailoff and Sawtschenko, 1910, Zell, 1913). Animal brain antigen may also be used (Marie and Urbain, 1929, Greval, 1932–3). Using a cocto-antigen, Kraus and Takaki (1926) obtained positive fixation with rabbit immune serum Kraus and Michalka (1926) substituted a glycerolited antigen instead of the cocto-antigen, and rendered the reaction more specific Shortt *et al* (1934–5) were also able to elicit a positive fixation reaction, and suggested that the method could be used to standardize antirabic serum Kostrezewski (1920) obtained a positive reaction with the sera of treated patients, using a cord antigen

Recently Casals has popularized the use of the CFT (Casals, 1942, 1947, Casals and Palacios, 1941 *a*, *b*) Antigen can be prepared from brain rendered noninfective by freezing and thawing or ultraviolet light, complement fixing antibodies develop to high titer in rabbits, mice, guinea-pigs, and dogs immunized with virus Antigen prepared by desiccating brain over sulfuric acid, and suspending in saline, can be used (Bernkopf and Nachtigal, 1943, Nachtigal, 1945). Nachtigal (1945) has claimed that the complement fixing antigen is "soluble" and occurs in the supernatant of centrifuged suspensions of infected brain

Despite these results, the occurrence of complement fixation in rabies has been denied (Baroni, Cinca, and Ionescu-Mihaiesti, 1908, Schultz, Bullock, and Brewer, 1928, Stuart and Krikorian, 1929–30)

Monkeys developing rabic infection were found to show no complement fixing antibodies although the virus neutralizing titer was high (Habel, 1945) Nachtigal (1945) also found a lack of correlation between the two antibodies, *in guinea-pigs*

The rabicidal antibody Many authors have demonstrated the development of rabicidal properties in the serum of animals treated by live or killed vaccines or tissue cultures, by the usual type of virus neutralization test (see, e g, Babès, 1891, Kraus, Kellner, and Clairmont, 1902; Schnurer, 1905, Marie, 1905 *a*, 1908; Semple, 1908, Kondo, 1922, Stuart and Krikorian, 1925, 1929–30, 1932 *a*, Jonnesco, 1939 *b*,

munized with normal central nervous tissue. Similarly, injections of normal rabbit brain did not produce rabicidal antibodies (Remlinger, 1909 *b*, Kondo, 1922). Harvey and Acton (1922–3) immunized 2 sheep, one with *virus fixe* and the other with normal rabbit brain. Although some antirabic properties developed after inoculation of normal brain, these were not so powerful as those produced by injection of *virus fixe*. Stuart and Krikorian (1929–30) were unable to stimulate the development of rabicidal antibodies in rabbits treated with normal nervous tissue

Thus far the specificity of the response appears to be proved However, the specificity of rabicidal serum has been questioned by Babès and Simici. They found (1910 *a*) that mice could be passively immunized (after infection) with the serum of a dog that had been injected with normal nervous tissue Further (1910 *b*), they stated that mice could be immunized by injections of normal central nervous tissue (Repetto, 1908, made similar observations) Despite these adverse observations, it is generally accepted that the rabicidal antibody is produced as a specific response to the injection of the virus of rabies *per se*.

H. THE NATURE OF ANTIRABIC IMMUNITY

From what has been said above, it will be evident that animals can be fairly readily immunized by injections of live or killed virus so as to withstand even an intracerebral test dose. Although the acquisition of this immune state may be accompanied by the development of serum antibodies, this is not invariable It would appear that virus fairly rapidly disappears from the site of inoculation in immune animals, probably due to an antiviral property of the tissues, and does not invade the nervous system. It seems reasonable to suggest that both humoral and tissue neutralizing substances are concerned in the destruction of virus introduced into the immunized animal

It is not nearly so easy to prevent the development of rabies if treatment is delayed until after the infection has been initiated. A beneficial effect can, however, be achieved by potent vaccines, vaccines plus serum, or serum alone. Serum presumably acts by a direct neutralizing effect on the virus before it invades the nerves Vaccination must in some way immunize the nervous tissues, so that the infecting virus fails to progress, but the precise mechanism is obscure. It may be that vaccines in some way occupy the susceptible cells before the arrival of the infecting virus, thus causing an "interference" phenomenon.

mareff and Solovieff, 1928). Hoyt and his coworkers have reported that antirabic (*rabbit*) *serum can protect mice when it is injected before an intracerebral test* dose of virus (Hoyt *et al*, 1936, Hoyt and Gurley, 1937–8, 1938). Further, they showed that injections of serum given after the test dose, even up to 4 days, still protected. A similar preventive effect of antirabic serum was observed if mice were *injected in the tail* (Proca, Bobes, and Jonnesco, 1935), or intramuscularly (Yen, 1942).

After inoculation of the pad in guinea-pigs, injections of sheep antirabic serum were shown to have a definite preventive effect (Proca, Bobes, and Jonnesco, 1934 *a*, *c*).

It has been found that intraspinal injections of serum, given just before or just after inoculation of virus, protect dogs and rabbits (Speransky, 1927; Ponomareff and Tchechkoff, 1927). The intraspinal injection was given after withdrawing *cerebrospinal fluid. If lumbar puncture was first carried out, intravenous injection* also protected these animals It is probable that this technique acted by altering the blood-brain barrier so as to allow the antibody to reach the nerve tissue. Intravenous injections of thionin have been found to have a similar effect on the blood-*brain barrier, and after such administration, antirabic serum injections may have a* powerful prophylactic effect (Loeffler and Schweinburg, 1932 *b*). Friedemann *et al.* (1944) injected virus intraventricularly in guinea-pigs, and administered serum intravenously, and found that there was a definite protective effect with adequate doses of serum.

Important experiments were carried out by Habel (1945) in guinea-pigs and mice Serum treatment of infected animals gave consistently better results than vaccination alone. Serum was most effective given intramuscularly at the site of inoculation. The protection was more effective if the serum was given immediately after the inoculation of virus. After infection with street virus, the most effective means of preventing rabies was to give serum up to 3 days later, and begin vaccine treatment 6 days after the administration of serum.

G. THE SPECIFICITY OF RABIC IMMUNITY

1 *The specificity of resistance to infection* It is generally held that the resistance of animals to infection after injection of *virus fixe* is a specific response to the virus Certain workers, however, have claimed that normal nervous tissue is capable of conferring antirabic immunity Thus Babès (1892, 1898) claimed that normal sheep's brain rendered dogs resistant to injections of street or fixed virus Fermi (1909 *b*) also advanced the antigenic potency of normal nerve tissue.

Against these observations, Remlinger (1909 *a*) was unable to immunize rabbits with normal homologous brain Harvey and Acton (1922–3) failed to protect dogs, monkeys, or guinea-pigs against virus by treatment with normal cord (prepared as in Pasteur's or Hoegyes's method) or normal brain (carbolized). Stuart and Krikorian (1929–30) were unable to confer any antirabic immunity on animals with carbolized normal brain.

2 *The specificity of the rabicidal antibody* First, the normal serum of cattle, horses, sheep, dogs, rabbits, guinea-pigs, and fowls is not rabicidal (Kraus, Kellner, and Clairmont, 1902, Kondo, 1922).

Secondly, as Harvey and Acton (1922–3) pointed out, the development of antirabic properties after injection of *virus fixe* might, theoretically, be due to the following causes

(*a*) A specific antibody response to the rabies antigen

(*b*) An antibody response to a nonspecific toxic constituent of the brain infected with fixed virus

(*c*) The production of a neurolysin in response to the injected brain

Various attempts have been made to exclude the 2nd and 3rd possibilities. Marie (1904 *b*) found that rabicidal properties did not develop when sheep were im-

ized vaccines It might, therefore, seem as if these vaccines should rapidly have supplanted live vaccine. No such result was, however, achieved, largely owing to the belief of the French that inactivated vaccines were inferior immunizing agents to living preparations At the Rabies Conference in 1927, for example, Calmette declared the superiority of living vaccines, there the position might have remained, but for the publication of numerous detailed statistical analyses by McKendrick This worker has shown that there is no difference in the immunizing capabilities of living or dead vaccines, when given in approximate dosage. These statistical reviews

linger and Bailly, 1939 *a*, Lépine, 1946).

INDICATIONS FOR ANTIRABIES THERAPY

Antirabies treatment should be started immediately when a person is bitten or scratched under the following conditions (Johnson, 1943) (1) The animal presents clinical signs of rabies (2) The animal is proved positive for rabies by laboratory

1940)

The amount of treatment is dependent on the degree of risk, and this is conveniently assessed by reference to Hempt's, or the newer Indian classification given on p 870

TISSUE USED IN ANTIRABIES VACCINES

Vaccine is usually prepared from rabbit brain or cord infected with *virus fixe* Sheep brain vaccine (5 per cent carbolized) is used in India and elsewhere, and has a higher antigenic value than sheep cord vaccine (Shortt *et al*, 1934, Covell *et al*, 1936–7, Dunlap, 1943) Monkey brain has also been used for treatment, e g, by the Hoegyes method (van Stockum, 1935, 1941) It has been found that removal of brain tissue from the vaccine lowers its immunizing value (Taylor and Menon, 1930). It may eventually prove possible to produce a vaccine for human use from tissue cultures

Golovine (1941) made the suggestion that if no vaccine could be obtained one could be prepared from the brain of the biting animal in an emergency

PROPERTIES OF *VIRUS FIXE* IN ANTIRABIES VACCINES

Choice of *Virus Fixe*

It is generally agreed that the classical Paris strain of *virus fixe* is the best immunizing agent It has been shown experimentally (Cunningham, Malone, and Craighead, 1933, Shortt *et al*, 1937), and in man (Shortt *et al*, 1934), that the Paris strain is a better immunizing agent than Kasauli and other Indian strains of *virus fixe*

strains passed more frequently were more highly antigenic The number of passages removed from the original Pasteur strain had no relationship to immunizing ability, yet of 5 strains fixed more recently, 3 gave a very high degree of protection Hampil and Roberts (1942), in similar studies in mice, found that substrains of the same

CHAPTER LXXVIII

HUMAN ANTIRABIES TREATMENT

HISTORICAL

In 1884 Pasteur, Chamberland, and Roux[1] reported that they had been able to immunize some 23 dogs experimentally against rabic infection. By the following year a method had been evolved which was capable of being applied to human beings. Thus, Pasteur (1885) reported that the cords of rabbits infected with *virus fixe* could be so treated that the virus became of greatly reduced virulence. Briefly, cords were suspended in dry air over potash, and the longer they were left to desiccate the less virulent they became.

On July 6, 1885, Joseph Meister, aged 9 years, arrived in Paris (see Pasteur, 1885). He had been badly bitten by a rabid dog on July 4, numerous wounds were situated on the hand, arm, and thigh. Although the wounds had been cauterized, Pasteur held that the child was doomed to die, and he injected the boy that day with a cord that had been desiccated for 14 days, the injection was given subcutaneously in the abdomen. The treatment was continued as follows: 2nd day, 14-day cord and 12-day cord, 3rd day, 11-day cord and 9-day cord, 4th day, 8-day cord, 5th day, 7-day cord, 6th day, 6-day cord, 7th day, 5-day cord, 8th day, 4-day cord, 9th day, 3-day cord, 10th day, 2-day cord, and finally on the 11th day, Joseph was injected with a fully virulent 1-day cord. Joseph remained in perfect health, and grew up to become the *concierge* of the Pasteur Institute.

The 2nd case was treated on October 20, 1885, the patient having been bitten 6 days previously, no rabies developed. By the 25th of February, 1886, Pasteur reported (1886 *a*) that he had administered his 350th prophylactic treatment, by the 12th of April, 726 had been treated (Pasteur, 1886 *b*). Of 688 cases treated after a dog bite only 1 died, whereas 3 of 38 persons bitten by a wolf were not saved by the treatment. By the 31st of October 1886, no less than 2,490 persons had undergone a course of prophylaxis. The treatment lasted for 6 days, beginning with a 14-day and ending with a 5-day cord. In this series there were 12 deaths (Pasteur, 1886 *c*). The actual plan of injections was modified somewhat with the passage of time (*vide infra*).

The treatment aroused the greatest possible interest in medical circles. Although some antagonistic communications appeared (see, e g, Dolan, 1886, Spitza, 1886), the general opinion was most favorable. In 1887 a number of medical men in Britain prepared a report and expressed themselves satisfied (*Rep loc Govt Bd*, 1887).

The 50th anniversary of the introduction of the treatment was suitably commemorated in Paris in 1935. A special supplement to the *Annales de l'Institut Pasteur* for 1935 was published, containing papers on antirabies treatment by Vallery-Radot (1935) and Lépine and Cruveilhier (1935 *a*), as well as many other articles on rabies.

Pasteur's original method has always been the method of choice in France and her colonies, in other parts of the world, however, numerous modifications have been introduced. Thus Hoegyes, in 1887, reduced the virulence of *virus fixe* by dilution. Both these vaccines contained, of course, living virus. From 1907 to 1911 phenolized vaccines were introduced by Fermi, and later modified by Semple. These vaccines contain inactivated virus—a radical difference from the earlier vaccines.

Although usually harmless, antirabies treatment has occasionally, from the earliest days, been followed by severe and sometimes fatal accidents. It was soon found that the incidence of such reactions was very considerably reduced by the use of phenol-

[1] References are appended at the conclusion of Ch. LXXIX, p. 887 et seq.

ized vaccines. It might, therefore, seem as if these vaccines should rapidly have supplanted live vaccine. No such result was, however, achieved, largely owing to the belief of the French that inactivated vaccines were inferior immunizing agents

example, Calmette de-

might have remained,

ilyses by McKendrick.

This worker has shown that there is no difference in the immunizing capabilities of

linger and Bailly, 1939 *a*, Lépine, 1946).

INDICATIONS FOR ANTIRABIES THERAPY

Antirabies treatment should be started immediately when a person is bitten or scratched under the following conditions (Johnson, 1943) (1) The animal presents

1940)

The amount of treatment is dependent on the degree of risk, and this is conveniently assessed by reference to Hempt's, or the newer Indian classification given on p 870

TISSUE USED IN ANTIRABIES VACCINES

Vaccine is usually prepared from rabbit brain or cord infected with *virus fixe*,

and elsewhere, and has

al, 1934, Covell *et al*,

treatment, e.g, by the

Hoegyes method (van Stockum, 1935, 1941) It has been found that removal of brain tissue from the vaccine lowers its immunizing value (Taylor and Menon, 1930). It may eventually prove possible to produce a vaccine for human use from tissue cultures.

Golovine (1941) made the suggestion that if no vaccine could be obtained one could be prepared from the brain of the biting animal in an emergency.

PROPERTIES OF *VIRUS FIXE* IN ANTIRABIES VACCINES

Choice of *Virus Fixe*

It is generally agreed that the classical Paris strain of *virus fixe* is the best immunizing agent It has been shown experimentally (Cunningham, Malone, and Craighead, 1933, Shortt *et al*, 1937), and in man (Shortt *et al*, 1934), that the Paris strain is a better immunizing agent than Kasauli and other Indian strains of *virus fixe* Habel (1940 *b*) studied 31 strains, 25 being derived from Pasteur's original He found a wide range in immunizing ability in mice in these strains. Apparently a high immunizing potency against a heterologous test virus is not an inherent characteristic of a

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and Roberts (1942), in similar studies in mice, found that substrains of the same

strain might vary considerably, and be weakly antigenic even against a test injection of homologous virus.

From what has been said it will be evident that the properties of *virus fixe* should be carefully checked from time to time (see below).

Alterations in the Properties of *Virus Fixe*

Many strains have been passaged since about 1886, and it is natural to inquire whether they have undergone any change that might affect their use in antirabies treatment. This question has been studied particularly by Remlinger, and is summarized in a review (1935 *a*), and also in papers with Bailly (1935 *a, b, c*), and by Habel (1940 *b*).

(*a*) *Virulence of fresh* virus fixe. Certain changes have been recorded in the length of the incubation period in animals. Thus, examining the Lille strain in 1930, Grysez and Marneffe (1935) found that the 6-day incubation period of earlier days had become replaced quite frequently by a 5-day period. Habel (1940 *b*) found that the period varied from 4–13 days with different strains.

Discussion has taken place whether fresh *virus fixe* has lost its pathogenicity to certain animals, but, using the intra-ocular route in rabbits, Marie (1923, 1927 *a*) found the Paris strain as virulent as previous. Contrary results have, however, been obtained by others Thus, Puntoni (1922) reported that the strain of *virus fixe* in Rome had become nonpathogenic on intraocular injection; Plantureux (1928) reported that the Algiers strain was losing virulence by this route, although 2 years previously it had been active (1926 *a*). The Tangier strain was found to be inactive by intraocular injection (Remlinger and Bailly, 1927 *c*)

Habel (1940 *b*) found all but one of his strains to be infective for mice by the intramuscular route. We ourselves have studied the properties of a British strain of *virus fixe* passed in guinea-pigs for many years. This strain usually infects by the intramuscular route only at a dilution of 10^{-1}. Tested by the intraocular route it behaves irregularly and occasionally infects at 10^{-3}.

(*b*) [illegible] passage, certain strains r[illegible] 13) noted that there w[illegible] changed over to a newly fixed virus, and this resulted in a lowering of the mortality. However. [illegible]

[illegible] 1940 *b*).

(*c*) *Resistance to desiccation* In 1923 Remlinger (1923 *c*) stated that the Tangier strain (2,200th passage) was more susceptible to desiccation than formerly Thus, while 3-day cords were always virulent, 4-day cords were only virulent in 50 per cent of cases, and 5- and 6-day cords were avirulent. Somewhat similar results were reported by Bablet (1925) from Saigon By 1934 Remlinger found (see 1935 *a*) that 3-day cords were sometimes avirulent, and even on occasions 2-day cords In the early days of this strain 5- and 6-day cords had proved virulent, so a considerable increase in susceptibility had occurred. Similar results were reported from Paris, where even 1-day cords were sometimes avirulent (Lépine, Cruveilhier, and Sautter, 1935, Lépine and Cruveilhier, 1935 *a*)

Various other institutes have reported a simil[illegible] cation, although not all strains show this featu[illegible] Thus, the Lyons strain in 1927 was still viruler[illegible] Bertoni (1938) reported that 5-day cords were still virulent at Boulogne, Virga [illegible] days' desiccation. Marie (1926) drew [illegible]sed as the test animals instead of rab- [illegible] Institute might prove virulent The

route of injection is also of importance in determining the possible avirulence of a ... was viru- ...) similarly ... n injected intraocularly and by other routes The most sensitive method of testing for live virus is the intracerberal injection of mice (see below).

(d) *Infectivity on d* ... *virus fixe.* Thus, in 188 ... lent, but later Remlinge ... *fixe* was active. Habel ... hundred-fold when tested in mice, ranging from 10^{-3} to 10^{-5}.

(e) *Resistance to glycerol* When this method was introduced by Calmette (1891) virulence was retained for about 1 month, even if cords had been previously desiccated for a few days The effect of passage on the Tangier strain was to render it more susceptible to glycerol, so that 2- to 3-day desiccated cords lost virulence after only 7 to 8 days' immersion (Remlinger, 1923 *d*, 1935 *a*) Rochaix (1923) reported a similar loss of resistance in the Lyons strain of *virus fixe*, and Grysez (1937) in that of Lille Not all strains of virus, however, have shown this increased susceptibility. For instance, 2-day cords infected with the Tunis *virus fixe* retain virulence in glycerol for up to 9 weeks (Burnet, 1925).

It must be remembered that the temperature of storage plays an important rôle in the duration of infectivity, virulence being retained best at low temperature. To quote an actual example, it was found that 1-, 2-, and 3-day cords remained virulent for at least 20 days at a temperature not over 10° C When, however, the temperature was raised to 20° C. virulence only lasted for less than 10 days (Girard and Robic, 1927)

(f) *Resistance to ether* With continued passage various *virus fixe* strains have been found to develop an increased susceptibility to ether. Thus Remlinger, Palmowitch, and Bailly (1931 *c*) studied the following strains of *virus fixe*, namely virus Z, Chisinau, and virus of *mal de caderas;* they were all rendered avirulent after 120 hours, whereas previously destruction had taken 144 hours. They reported a similar increased susceptibility for another virus strain (B).

However, the results of ether resistance tests must be regarded as somewhat variable In 1934–5 Remlinger (see 1935 *a*) repeated his original (1919) work on the resistance of Tangier *virus fixe* infected brains to ether, he found that such brains now resisted treatment for 250 to 280 hours, although previously 120 to 125 hours had sufficed for destruction.

In conclusion, therefore, the effect of continued passage on *virus fixe* has been to increase its susceptibility to desiccation and glycerol, but to render it more resistant, certainly to dilution and in some cases to ether as well

Preservation of Virulent Fixed Virus

1. The first method introduced to meet this need was glycerol (Roux, 1887, Calmette, 1891, Rodet and Galavielle, 1901 *a*, 1902, Nicolle, 1904) Habel (1940 *b*) found that 4 strains lost their infectivity after 3 months in 50 per cent glycerol at 0° C., 4 were viable up to 6 months, and 23 for 6 months or longer. When brain tissue is immersed in glycerol, infectivity is lost from the periphery inwards, and therefore the larger the piece of tissue the longer it remains virulent (see Remlinger, 1924 *a*). Further, the higher the temperature at which storage occurs, the quicker is virulence lost (Protopopoff, 1889)

... not ... loc ... undesirable ingredients of a human vaccine these organisms may eventually cause the virus to die out (Remlinger, 1923 *b*)

2. A better method is cold storage, as virus retains its virulence in the cold (+4° C. to −10° C.) for a long time, especially when frozen (Viala, 1891; Puntoni, 1924; Stuart and Krikorian, 1929–30, Remlinger and Bailly, 1934 *b*, Lépine, 1935). Under these conditions virus *may retain its virulence for a year or longer. Storage* in a carbon dioxide ice box is the method of choice.

3. Virus rapidly desiccated preserves its virulence for some months (Vansteenberghe, 1903, Baroni, 1927).

4. Although virus maintains its virulence in olive oil for some weeks (Botafogo Gonsalves, 1924), this treatment renders the brain soft, disintegrated, and scarcely suitable for use in antirabies treatment (Remlinger, 1924 *c*)

5. Formol serum, phenol serum, and formol glycerol serum have been said to preserve virulence for about 3 weeks (Plantureux, 1925 *a*, *b*), Remlinger and Bailly (1925) regard the method as inferior to glycerol for antirabies vaccines

Maintenance of Titer of Fixed Virus

With any one strain of fixed virus the greater the amount of virus material the greater the immunizing capacity of the vaccine. It is, therefore, desirable to insure the maximal titer in the brain used as source of inoculum. Habel (1941 *a*) recommends that rabbits be inoculated intracerebrally with 0.2–0 25 c.c. of a 1/1000 dilution of supernatant fluid from the suspension of a previously passed brain The animal should be killed after it has been completely paralyzed for at least one and preferably two days After killing by chloroform, or exsanguination, the brain may be kept for a short time in glycerol or at −10° C. The cord should be discarded. Frequency of passage does not influence the final titer in the paralyzed animal (see also Remlinger and Bailly, 1935 *a*).

Periodical Checking of Properties of *Virus Fixe*

Laboratories preparing antirabies vaccines should periodically test the properties of their strain, along the following lines recommended by Habel (1940 *b*) The specificity of the virus should be checked by neutralization tests with known rabies antiserum Samples of virus should be removed at intervals during the process of treatment with chemicals and tested for virulence in mice. Rabbit brains should be examined for virus content by titration in mice Mice should be immunized with the final vaccine, and challenged with a known rabies virus When live vaccines are being used, in addition, the infectivity for mice by the subcutaneous route should be estimated. Also, in the Pasteur method, samples of cord desiccated for varying periods should be tested by mouse inoculation for virulence.

TESTING OF VACCINES FOR VIRULENCE

Chemically prepared vaccine must be tested by animal inoculation. Advantage should be taken of the susceptibility of the mouse, and at least 5 2-week-old mice injected intracerebrally with 0 03 c.c. of a 1:10 dilution of the vaccine (Webster, 1939 *b*)

ROUTE OF INJECTION

The vaccine is usually injected subcutaneously, often in the abdomen The intravenous route has been recommended (Téodorasco, 1923 *a*, *b*; Morison, 1924) but is seldom used Intradermal injection is also practiced

A. VACCINES IN COMMON USE

In the following pages we shall discuss those methods which feature in Greenwood's (1945–6) statistical review of antirabies treatments, that is to say, those methods which are being used to a greater or less extent by various antirabies institutes throughout the world These methods are as follows [1]

[1] The numbers used in referring to these vaccines are those employed by Greenwood (1945–6)

1. Desiccated cord method and modifications (Pasteur and Calmette)
2 Hoegyes's dilution method
3–6 Phenol methods–modifications of Fermi's method, viz
   3. Killed phenol methods (Semple and Mulford)
   4 Live phenol (Puntoni's) method
   5. Fermi's original method
   6. Fermi's serovaccine
7 Heated nerve tissue methods (Babès)
8 Killed ether (Hempt's) method
9. Mixed methods
   (a) dilutions plus, in severe cases, Alivisatos's ether method
   (b) dried cords plus, in severe cases, Fermi's original method (5)
   (c) Fermi's vaccine plus, in severe cases, Alivisatos's

These vaccines may also be grouped under four headings as follows
(a) Killed vaccines (3 and 8)
(b) Live vaccines (1, 2, 4, 9 a, b)
(c) Heated vaccines (7)
(d) Other vaccines (5, 6, 9 c, 10)

1. Desiccated Cord Method

The effect of desiccation on fixed virus It was realized from the start that virulence was usually lost in cords after about 5 to 6 days of desiccation over potash, 5- and 6-day cords usually being noninfective, while 4-day cords were virulent (see, e g, Gamaleia, 1887 *d*, Rodet and Galavielle, 1901 *b*). The curve of infectivity of cords subjected to desiccation falls rapidly during the first 3 days, and very slowly thereafter to the 9th day, the loss of infectivity was found to be directly proportional to the rate at which the contained moisture is lost (Harvey and McKendrick, 1907)

The exact loss of virulence that occurs with desiccation is greatly affected by the temperature Thus, other things being equal, cords desiccated at the usual 23° C lose virulence quicker than cords treated at 4° C. There seems to be considerable variation between different institutes with regard to the temperature used for desiccation, and Remlinger (1925 *d*) has entered a plea for greater uniformity.

The fact that with continued passage certain strains of *virus fixe* are now very much more susceptible to desiccation than formerly has already been mentioned In fact, Lépine (1938) has stated that it may become necessary to give up cord altogether.

Preparation of vaccine This method of antirabies treatment was, of course, originated at the Pasteur Institute in Paris, and is most widely used in France and her colonies We shall describe the method with particular reference to the present-day technique detailed by Lépine and Cruveilhier (1935 *a*) Reference will also be made to the practical information furnished by Harvey and Acton of the Kasauli Institute (1922–3) The strain of virus used in Paris for the preparation of vaccine is the original classical strain, it has been passaged continuously since the days of Pasteur Rabbits are most commonly used as experimental animals and are injected subdurally with *virus fixe* If dogs are being used for injection, the intracerebral route is preferable to the subdural In carrying out the subdural inoculation, the usual practice is to inject virus under the dura, from behind forwards, through a small trephine opening in the angle of the coronal and sagittal sutures but, if desired, the skull may be perforated with a sterile nail or drawing-pin (Remlinger, 1926 *a*)
To preve
(a) slow
with a sv
withdrawn slowly Care should be taken that antiseptics do not enter the needle hole

At the present day rabbits are killed when paralysis sets in (6th to 7th day), instead of, as formerly, waiting until they die. It was often found that the cord removed in such circumstances was contaminated with organisms (see, e g, Kuhne, 1921, Remlinger, 1923 *a*, Isabolinsky, 1924). Cords are best removed by cutting the vertebral column across high up and low down, and pushing the cord out (from below upwards) with a sterile nickel rod. The cords should be received into sterile receptacles, and routine cultures made. Each cord is cut into 2 or 3 pieces and suspended by a sterile wire in tubes in a special flask with caustic potash at the bottom. The flasks are then incubated at 23° C. When the desire length of time has expired, the cords are placed in glycerol and stored on ice.

Human inoculation. A typical course of injections with desiccated cord, as used at the present day, is shown in Table 33. Each dose consists of about 3 to 4 mm of cord emulsified in 3 c.c. of sterile water.

TABLE 33 [1]

SPECIMEN PRESENT-DAY COURSE OF ANTI-RABIES TREATMENT WITH DESICCATED CORDS

Day of Treatment	*Injections*	*Day of Treatment*	*Injections*
1	4-day cord	14	3-day cord
2	4 " "	15	2 " "
3	4 " "	16	3 " "
4	4 " "	17	3 " "
5	3 " "	18	2 " "
6	3 " "	19	3 " "
7	4 " "	20	2 " "
8	3 " "	21	2 " "
9	2 " "	22	2 " "
10	3 " "	23	2 " "
11	3 " "	24	2 " "
12	2 " "	25	2 " "
13	3 " "		

[1] Modified from Lépine and Cruveilhier (1935 *a*) in accordance with a personal communication from Dr Lépine (July 1946).

Cases least at risk are treated for 15 days, average cases for 18 days, more serious cases for 21 days, and the most serious cases for 25 days.

Modifications of the desiccated cord method.

(*a*) *Glycerol modification.* Antirabies treatment can be carried out with cords which have remained in glycerol for decreasing periods of time, without being desiccated at all Thus, the first injections consist of cord which has been in glycerol for about a month. It has long been known that such glycerolated cords can confer immunity on animals (Rodet and Galavielle, 1901 *c*, Galavielle and Martin, 1902, Rodet, 1924)

Recently some modification of the older methods has been necessitated owing to the increased susceptibility of certain strains of *virus fixe* to glycerol Thus, Grysez (1937) of Lille recommends that the course be begun with cords preserved at +23° C for 9 to 16 days, and continued with cords glycerolated for 1 to 8 days As Oláh (1938) has reported that *virus fixe* is more resistant to phenol-glycerol than to glycerol it is possible that some such method of attenuating the virus may be introduced

(*b*) *Funayama's (1934) modification* This worker used an intensive course which began with 5-day cords and ended on the 18th day with a 1-day cord

(*c*) *Nicolle's modification* (see Marie, 1927 *b*). The treatment consists of 3 courses of injections.

World use of desiccated cord method.

Dried and glycerolated cord methods are extensively used in France, the French colonies, and other French-speaking countries, also in Alexandria, Bangkok, Berne, Brazil, Argentine, Chosen, Montevideo, Rio de Janeiro, Tokio, Poland

A report of the year's work of the Pasteur Institute of Paris appears yearly in the *Annals de L'Institut Pasteur* (60, 661; 62, 661; 64, 565; 66, 483; 68, 545; 69, 371, 71, 34, 294, 72, 952; 73, 1191).

Method 9 (*b*), dried cords plus Fermi's vaccine, is used in Buenos Aires (according to McKendrick, 1940).

2. Hoegyes's Method

Experimental. This method was introduced by Hoegyes in 1887 (see also [illegible] injections [illegible] last for a [illegible] ubinfective dilutions and gradually increased the strength until fully virulent doses were administered. Hoegyes's original dilutions were usually fully infective up to 1·200, 1.5,000 might be infective, while 1.10,000 was not infective. Weaker dilutions do not kill the animal more slowly than stronger, if the animal dies at all following injection of a dilution it does so in the usual time (Remlinger, Palmowitch, and Bailly, 1931 *d*) We, however, have noted a considerable prolongation of the incubation period, especially in guinea-pigs injected with weaker dilutions. The following correspondence has been observed between the infectivity of dilutions and desiccated cords (see Marie, 1927 *b*)· 1 10,000–1·6,000 dilution corresponds to 14- to 8-day cords, 1.5,000 to 7-day cords, 1·1,000 to 6-day cords, 1·500 to 5-day cords, 1 200 to 4-day cords. It should be noted, however, that certain strains have been found to show a greatly increased infectivity after many years' passage, and it is probable that these original figures are now inaccurate.

This method of treatment has been widely used in human practice and has proved very efficacious. Webster (1939 *b*) found the method to be effective in immunizing mice

Human inoculation The numerous modifications in dosage which this treatment has undergone since its inception need not be detailed, and Marie [illegible]

3–6. Phenol Methods (Modifications of Fermi's Method)

These vaccines have been very widely adopted in antirabies practice The virus may sometimes be definitely killed by the antiseptic, in other cases the vaccine contains attenuated virus

The effect of phenol on fixed virus The lethal effect of phenol on *virus fixe* had been known for many years before phenolized vaccines were actually introduced (see, e g, de Blasi and Travalli, 1890) The originators of the various methods (*vide infra*) have all studied the effect of phenol on fixed virus (see also Sawtschenko, 1911, Cumming, 1914) Although there is a certain amount of variation in their findings, it appears that in 0 5 per cent phenol a 1 per cent dilution of virus remains viable for at least 20 days at 20° C, 1 per cent. phenol will destroy a 1 to 2 per cent suspension of infected brain in 4 to 7 days at 20° C. (Semple, 1911). At 37° C. 1 per cent phenol will destroy up to an 8 per cent. suspension of virus in 24 hours (Semple), and a 20 per cent suspension in 144 hours (Stuart and Krikorian, 1929–30) Habel (1940 *b*) who worked with 31 strains of *virus fixe*, obtained somewhat different results At 37° C. he found that while some strains were destroyed

by 1 per cent. phenol in under 24 hours, others were still viable after 4 days He found that the longer the strain resisted phenol the greater the immunizing capacity.

Phenolized vaccines are prepared by adding phenol, usually 1 per cent, to infected brain tissue and leaving in the 37° C. incubator for about 4 days. At this stage, Habel (1940 *b*) found that most vaccines still contain viable virus The vaccines are then usually diluted to contain 0.5 per cent. phenol, and stored in the ice chest. Habel (1940 *b*) did not find any viable virus after 2 months storage

It appears that in the U S A. a number of phenolized vaccines exposed for sale for the treatment of man has been devoid of immunizing potency when tested in mice (Webster 1939 *b*).

Keeping properties. One of the great advantages of these vaccines is their keeping properties. For instance, it was found (Gloster *et al*, 1925–6) that carbolized vaccine retained a high degree of immunizing potency whether kept in the cold room or at shade temperature in Rangoon (approx. 87° F.) Vaccine was found to remain potent for at least 7 months after storage in an ice-chest in the plains of India in the summer (Covell *et al*, 1936–7).

3. Killed Phenol Methods

(*a*) *Semple's* (*1911*) *modification.*

Experimental. This method was originally introduced for use in India, and has been widely adopted in many parts of the world. For use as a vaccine, an 8 per cent dilution of brain in 1 per cent. phenol in normal saline is incubated for 24 hours at 37° C, and then diluted with normal saline to make a 5 per cent. dilution The vaccine is kept at room temperature for at least 30 days before use (*Report*, 1938–9)

Semple was able to immunize monkeys, dogs, and rabbits by subcutaneous injections of his vaccine Webster (1936) carried out a series of experiments in which he showed that mice could also be immunized by intraperitoneal injection. Habel (1940 *b*) examining the immunizing capacity in mice of phenolized vaccines prepared from 31 strains, found that 12 were quite ineffective, 10 moderately efficient, and 9 strains immunized against at least 2000 MLD

Human inoculation. Semple's vaccine (5 per cent. brain in ½ per cent phenol) has been widely used, both in India and elsewhere The dosage recommended by the Pasteur Institute at Kasauli depends on the Classification of Risk (see p 870) and is as follows Class I, all cases, 2 c.c. daily for 7 days, II, 5 c c for 14 days, III, adults, 10 c c daily for 14 days, children, 5 c.c. daily for 14 days (Webster, 1944).

World use The method is in use in West Africa, Ankara, Baghdad, Bangkok, India, Brazzaville, Roumania, Ceylon, Poland, Hong Kong, Malaya, Lisbon, Kenya, U S A, Canada, Palestine, Burma, Chile, Cuba, Shanghai, Teheran (McKendrick, 1940).

(*b*) *Stuart and Krikorian's* (*1925*) *modification*

This vaccine is a slight modification of Semple's, containing less nerve tissue It consists of a 2 per cent emulsion of virus-infected brain killed by 1 per cent. phenol at 37° C for over 24 hours. Before use it is diluted with equal parts of normal saline. Experimentally, rats and rabbits have been protected by this vaccine For human use, 5 c c is injected daily for 14 days intracutaneously in the abdomen This method has been widely used in Palestine (see Stuart and Krikorian, 1929–30).

(*c*) *Phenolized vaccines and antirabic serum*

It has been suggested that antirabic (sheep) serum be used along with carbolized vaccines (Shortt *et al*, 1934–5)

4. Live Phenol (Puntoni's) Method

In Puntoni's method the infected nervous tissue is ground up with 1 per cent. phenol (1921 *a*, *b*, 1923, 1928). After about a week at 21° C. this suspension is avirulent Doses of increasing virulence are inoculated, and from 40 to 100 injections may be given

Puntoni's method is used in Florence and Brazil

5, 6, 9 *b*. Fermi's Method (see Fermi, 1909 *a*, *b*, *c*, 1925, 1934)

In Fermi's original method (5) *virus fixe* was treated with 1.0 per cent. phenol, which was said to sterilize a 5 per cent virus suspension. Babès and Bobes (1908) found, however, that such treatment might fail to destroy the virus The usual course of treatment comprises daily injections of 5 to 6 c c. for 3 weeks. In Fermi's serovaccine method (6) antirabic serum is combined with virus Fermi's method is sometimes employed as an adjuvant to the desiccated cord method (9 *b*).

Fermi's original vaccine is used in Buenos Aires, Czechoslovakia, Egypt, Budapest, Dakar, U.S S R , Mexico, and Bolivia

Fermi's serovaccine has not been used since 1934

Lépine and Sautter's (1937) modification.

These authors have recommended a vaccine consisting of a 5 per cent. rabbit brain suspension in 1 per cent. phenol-water. The vaccine may be used after 24 hours' attenuation at 20 to 21° C, it may be stored on ice for 2 months without significant loss of potency. Rabbits could be effectively rendered resistant by such a vaccine The method is used in Beirut and Teheran (Lépine, 1946), and in association with Pasteur's method in Paris, Algiers, Hanoi, Saigon, and Antanarivo

Advantages of Phenolized Vaccines

Phenolized vaccines are very widely used throughout the world Their advantages have been summarized as follows (Remlinger and Bailly, 1936 *d*)

(*a*) They are easily prepared in the laboratory, with a minimum of apparatus (see Lépine, 1937).
(*b*) As dog or sheep brain may be used, fewer animals need to be sacrificed than in the desiccated cord method
(*c*) The antigenic potency is conserved for several months
(*d*) The vaccine is easily rendered sterile, and it is simple to carry out appropriate bacterial sterility tests.
(*e*) A considerable reserve stock of vaccine of guaranteed and lasting potency can be maintained (see also Zeuner, 1943).
(*f*) The vaccine is readily transportable, and treatment can thus be decentralized Patients may, therefore, be treated earlier and saved a long journey to a central institute.
Carbolized vaccine is very useful for bodies of troops far from a Pasteur Institute (see, e g , Hamerton, 1922).
(*g*) Any medical man equipped with a syringe and needle can inject the vaccine, without special technical skill
(*h*) Phenolized vaccines produce the minimum number of cases of neuroparalytic accident.
(*i*) The tissue can be mechanically ground, thus producing a true emulsion.

7. Heated Nerve Tissue Methods

These methods are often known after the names of Babès and Puscariu (see, e.g , Babès, 1912 *a*, Puscariu and Lebell, 1914, Téodorasco, 1923 *b*) Heating destroys or attenuates virus according to the temperature employed. Puscariu and Vesesco

(1895) showed that heat-killed virus was still antigenic, and animals have been immunized by courses of such heated vaccines. At first the course was begun with cords heated to 80° C., and although these were noninfective, they were still antigenic (Babès and Lepp, 1889). The treatment was continued with cords heated to 75° C., 60° C., 55° C, 50° C, and 45° C; on the last day unheated cords were given. Later (after 1908) only cords heated at 65° C. were used.

Another modification of the heated cord method is the one used at the antirabies centers in Cluj (Roumania), as described in 1935 by Botez (see Botez and Albon, 1929, also Botez, 1930).

The heated cord methods are used in Roumania, sometimes combined with serotherapy (Proca and Bobes, 1940).

8 and *9 a*. Etherized Vaccines

Etherized vaccines have been used extensively in antirabies treatment (Alivisatos, 1922, 1926, Hempt, 1925; Busson, 1925, Da Silva, 1926 a). Remlinger (*1918 a*) was the first worker to suggest the use of this type of vaccine. As usually employed, these vaccines contain attenuated but not completely killed virus. Zeuner (1943) has shown that the ether content, although sufficient to destroy nonsporing bacteria may not destroy sporing organisms.

The effect of ether on fixed virus. Remlinger showed that after 120 hours in sulfuric ether, the brain of an infected rabbit was rendered quite avirulent Alivisatos (1922) reported that virulence was lost after 140 hours. Virulence is lost from the periphery inward, the center of the brain remaining virulent longest Thus, Remlinger (*1919 c*) found that the periphery had lost its infectivity in 16 hours while the center did not do so for 120 to 125 hours Correspondingly, the smaller the piece of brain immersed the quicker is virulence lost (Iyengar and Beer, 1930–1). Cords lose their virulence in ether very much more quickly than brains, being usually avirulent in under 12 hours. To render etherized vaccines avirulent phenol is sometimes added Virus is destroyed when 1 per cent. phenol is added to a brain that has been 72 hours in ether, and incubation carried out at 37° C (Iyengar and Beer, 1930–1).

Varying ether resistance of different strains. A particular study of the comparative resistance of the Pasteur Institute (Paris) and Indian strains of *virus fixe* has been made by workers in India (Cornwall and Beer, 1925–6 *d*, Cunningham, Nicholas, and Lahiri, 1926–7, 1928, 1928–9 *b, c*). They found that the Paris strain was resistant to ether and was not destroyed under 168 hours' immersion. With Kasauli strain K2 destruction of virus occurred after 84 hours. The classical Kasauli strain was usually destroyed or markedly attenuated after 36 hours' immersion, although destruction did not invariably occur after 84 hours It is evident, therefore, that Kasauli strains are much more easily destroyed by ether than the Pasteur Institute (Paris) strain

Antigenicity of etherized vaccines Animals have been rendered resistant by inoculations of virus attenuated by ether (see, e g, Remlinger, *1919 c*). It has been noted that, although Berkefeld filtrates of rabies virus immunize rabbits (Remlinger, *1903 a*), this property is lost when ether is added (Remlinger, *1919 c*)

Human inoculation Hempt's (1925) method (8) was once used quite extensively, etherized virus being diluted 1 60 in saline. In mild cases 1 to 2 gm were inoculated, and 3 to 4 gm in severer cases, injections were given twice daily for 4 to 6 days This method was later superseded by a killed ether (carbolized etherized) vaccine

The treatment is used in Yugoslavia and Prague

Alivisatos's (1922) method (9 *a*) This method is not widely used, the vaccine consisting of *virus fixe* treated with ether for 72 to 84 hours As Alivisatos found that the infectivity of brain was only destroyed after 140 hours in ether, the method evidently employs attenuated virus

At the present day Alivisatos's method is used in severe cases as an adjuvant to the dilution method, in Athens and Vienna and certain Bulgarian institutes (see McKendrick, 1940). Method 9 (*c*), Alivisatos's method plus Fermi's method, is used in Salonica.

10. Yatren Method

A vaccine prepared from nervous tissue treated with yatren was introduced by Abadjieff (1928, 1932). Okuwada (1933) has used the vaccine experimentally. Fixed virus was destroyed by 5 per cent yatren in 3 hours at 37° C. In vaccine preparation a 10 per cent emulsion of brain in 4 per cent yatren was incubated for 24 hours at 37° C, before use it was diluted 1 in 4 and filtered.

B. VACCINES LESS WIDELY USED

Vaccines Containing Virulent Virus

1. *Ferran's method* An intensive treatment was recommended by Ferran (1888), who gave daily injections of virulent bulb in water.

2. *Cumming's (1914) method* In this method a homogeneous brain suspension was dialyzed against running (distilled) water. Injections of this dialyzed vaccine rendered rabbits resistant to 8 MLD of *virus fixe* injected intracranially. This vaccine was used in the United States of America, 2 c c being injected daily for 15 to 25 days. An anaphylactic reaction often occurred on the 7th or 8th day.

3. *Harris's method* (1912, 1913 *a*, *b*) Harris found that the infectivity of rabies virus could be preserved for a considerable time by freezing the brain and cord of a rabbit with carbon dioxide snow or liquid air, and drying *in vacuo* Infectivity was also preserved for several months by drying *in vacuo* at −18° C For use as a vaccine, tissue frozen with CO_2 snow was pulverized in a mortar, dried *in vacuo* over sulfuric acid, sealed and stored in the ice chest. At −10° C. no loss of infectivity occurred for at least 2 years.

Writing in 1921 D'Aunoy reported that this method had been in use in New Orleans for 6 years Adults were given 11 doses totalling 17,750 minimal infecting doses of virus preserved by freezing In severely bitten persons, 15 doses totalling 25,750 minimal infecting doses were administered Webster (1939 *b*) found the method effective in mice

4 *Marie's method* (see, e g, 1927 *b*) In this method excess *virus fixe* is mixed with antirabic serum (sheep) The course is begun with daily subcutaneous injections of a mixture of 2 c c of a 1 10 suspension of virus and 4 c.c antirabic serum; on the 5th day the treatment is continued with 6-day desiccated cords

5 *Phillips's (1922) method* The rabbit's brain is removed and ground to a paste in a mortar, and glycerol is added so that 0 1 c.c. of suspension should contain 15 mg of fixed virus material This suspension is filled into glass ampules, which are placed in a test tube and stored in the cold for a few hours The mouth of the test tube is then packed with pyrogallic acid, and potassium hydroxide is added, the tubes are stored at −2° C to −4° C Virus remains living after this treatment, and is used for human inoculation after a 3-day course of dead *virus fixe* (phenolized) Lubinski (see Kraus, Gerlach, and Schweinburg, 1926), using a similarly prepared vaccine, gave 20 daily injections of 2 c c of various dilutions as follows 1st day—1 200, 2nd day—1 100, 3rd and 4th days—1 66, 5th and 6th days—1 40, 7th day—1 25, 8th to 10th days—1 66, 11th to 13th days—1 40, 14th day—1 25, 15th and 16th days—1 66, 17th to 19th days—1 40, 20th day—1 20 Various modifications of this method are in use in the USSR

6 *Proescher's method* (1909, 1911) A number of cases was treated with unchanged *virus fixe* The brain of an infected rabbit was preserved on the surface of a 50 per cent glycerol agar slope, when required an emulsion was made and

(1895) showed that heat-killed virus was still antigenic, and animals have been immunized by courses of such heated vaccines. At first the course was begun with cords heated to 80° C, and although these were noninfective, they were still antigenic (Babès and Lepp, 1889). The treatment was continued with cords heated to 75° C, 60° C, 55° C., 50° C., and 45° C.; on the last day unheated cords were given Later (after 1908) only cords heated at 65° C. were used.

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(*d*) When they wane, the titer can be increased by only a few more injections of cord.
(*e*) They found that the longer the course of treatment, the more pronounced the rabicidal power of the serum Correspondingly, the less desiccated the cords the higher is the rabicidal titer.

There is no definite unanimity with regard to the relationship of the rabicidal antibody to the protection acquired against rabies. It seems safe to conclude, however, that while protection and antibody formation usually occur together, this is not invariable. Thus, a person may be protected against rabies and yet show no

THE BLOOD DURING ANTIRABIES TREATMENT

Blood changes appear to be variable. Certain workers have referred to a leukopenia (Carvalho, 1922; Leger, 1923; Jonnesco, Valter, and Teodasiu, 1927), while others refer to a leukocytosis usually involving neutrophils and eosinophils, but sometimes lymphocytes and monocytes (Dodero and Hoang-Tich-Try, 1940). Some say the Arneth count is shifted to the right, others to the left.

Dodero (1940*b*) found that both upper and lower limits of fragility were usually lowered Heterophil hemagglutinins may develop (Rosenfeld and McDaniels, 1939).

MODE OF OPERATION OF ANTIRABIES TREATMENT

Curiously enough, th
important point: how
by Remlinger (1907*a*)
after it has reached the central nervous system. On the other hand, it may be that the injections serve to prevent the virus from reaching the central nervous system, certainly there would appear to be time to arrest the intraneural spread of virus,
may be
devel-
ijection
of animals with virus does not appear to confer immunity with any regularity, it would seem, nevertheless, that antirabies immunity is a tissue immunity. The cells of the central nervous system must, in some way, be rendered resistant to the rabies virus. It will be recalled that Negri bodies are less commonly found in treated persons who develop rabies than in untreated (Busson, 1930) It is possible that the interference phenomenon may be responsible for antirabic immunity. The susceptible cells may be "blocked" by the living particles of *virus fixe*, or inactivated particles, depending on the type of vaccine. When the street virus reaches the nervous system, it finds the susceptible cells already occupied. Or it is possible that the phenomenon of Magrassi (see Ch. XVII) may be concerned, the meeting of the two viruses in the brain leading to mutual extinction

DECENTRALIZATION OF ANTIRABIES TREATMENT

Patients bitten by a rabid or suspectedly rabid animal must be subjected to antirabies treatment as soon as possible. In countries where there is adequate and reasonably rapid means of transport, it is probably best to send the case to a central institute Here the patient can be treated by experts who devote their whole time to the treatment. Indeed, certain methods of treatment can be carried out only

0.01 gm. of brain tissue injected. In 5 days 0.05 gm. of virulent *virus fixe* was injected

7. *Krasmitski's (1902) method.* In this method filtered and diluted virus was heated to 37° C., and thus presumably slightly attenuated.

Vaccines Containing Dead Virus

1. *Lipovaccine.* It has been reported that the emulsification of virulent virus material in olive oil renders it avirulent. The emulsion is still antigenic, however (Botafogo Gonsalves, 1925).

2. *Formol method.* Cumming (1914) showed that 0.08 per cent. formaldehyde destroyed the virus in 2 hours. Formolized vaccines do not appear to have been widely used by public institutes, although they are supplied commercially in the United States Tzekhnovitzer and Goldenberg (1930) found formolized vaccines antigenic in experimental work. Shortt *et al.* (1937) showed that a formolized vaccine was as potent as a carbolized, in a series of animal experiments, while Jacotot *et al.* (1938) found, using guinea-pigs, that such vaccines were actually superior to phenolized vaccines. van Stockum (1938, 1941) recommended a 10 per cent. suspension of monkey brain formolized (1.5 per cent.) after incubating at 37° C. for 5 days.

3 *Purified vaccines.* Kaiser (1943) recommends a vaccine treated with ether to remove nonvirus nervous tissue, the virus being exposed later to iodine vapor. Veeraraghavan (1941) has purified sheep brain vaccine by iso-electric precipitation.

4. Sellers' (1923) vaccine was diluted, glycerolated, and phenolized.

The Use of Serum in Antirabies Treatment

Following the bite of a rabid animal, street virus probably remains at the site of the wound for a short time before invading the nervous system. When vaccines are given, even at once, there is bound to be a delay of several days before resistance develops It would, therefore, appear to be logical to administer antirabic serum locally and generally as soon after the bite as possible, to neutralize the virus before it can invade The results of serum treatment of experimentally infected animals have been most satisfactory (Habel, 1945, see p. 852). Antirabic serum should be combined with a course of active immunization (Shortt *et al*, 1934–5, Habel, 1945) Proca and Bobes (1940) have used serotherapy quite extensively and claim beneficial results They find that serotherapy tends to prolong the incubation period in cases that do develop rabies despite treatment. They accordingly suggest that a 2nd course of vaccine treatment be given about 2 months later

DEVELOPMENT OF RABICIDAL PROPERTIES IN THE SERUM AFTER ANTIRABIES TREATMENT

Many workers have recorded the development of rabicidal antibodies in the serum after a course of antirabies treatment (e g, Kraus and Kreissl, 1902, Semple, 1908, Kostrezewski, 1920, Puntoni, 1921 *a*, Nikolajewa, 1925, Alivisatos, 1926, Da Silva, 1926 *a, b, c*, Stuart and Krikorian, 1931–2, Webster, 1936). The development of these properties has been particularly studied after treatment with desiccated (or glycerolated) cords, and phenolized or etherized virus.

The findings of later workers, such as Cruveilhier and Viala (1937) and Dodero (1937 *a*), serve to corroborate the observations of many earlier workers, and are as follows

(*a*) After treatment with desiccated cord, rabicidal properties are found to develop in the serum

(*b*) These antibodies do not usually develop till about 10 days after the close of the antirabies treatment

(*c*) They remain present for up to 7 months

Incubation Period of Rabies in Treated Cases

Approximately one-third of cases develop their illness less than a month after the bite, one-third between 1–2 months, and the remaining third after 2 months. It has been stated that antirabies treatment may actually bring about death from rabies after a shorter incubation period than in untreated persons, as if *virus fixe* inhibits the defense mechanisms, and accelerates the passage of the street virus (see review of Proca and Bobes, 1940).

It has been suggested that rabies may develop in treated persons after an emotional "shock," as if the street virus was lying "latent" in the nervous system (Remlinger, 1945, 1946 *b*).

Cases of rabies developing after long incubation periods are said to be commoner when serum is used in treatment (Proca and Bobes, 1940).

Assessment and Presentation of Mortality Rates

The assessment of mortality from antirabies treatment. At first sight, it might appear that it would be a comparatively simple matter to record fatalities from antirabies treatment by following up all treated cases to see whether or not they remain in health. While this may be easy in European countries, it is extremely difficult in the East In India, for example, the patients return home after treatment, and it may be difficult to trace them again In the first year of operation at Kasauli, follow-up returns were received from 76 per cent. of treated patients, but in 1925 from only 30 per cent. On the introduction of improved methods, 80 per cent. of patients were followed up, and this resulted in a detection of more than double the previous number of deaths (Cunningham and Malone, 1930). Again, should a person die after antirabies treatment, it is possible that the attending doctor who signs the death certificate may be quite unaware of this fact and sign the certificate marked with some other cause of death There is no doubt that a proportion of fatalities after antirabies treatment is not recorded.

The presentation of rabies mortality rates. A practice that used to be adopted by antirabies institutes was to give what they called the "failure rate" These were cases in which rabic symptoms developed within about 15 days after the termination of the treatment. This rate is fallacious, as the shorter the treatment, the less cases will develop within this 15 days, conversely, the longer the treatment, the more cases will be included. It is far more accurate to give the total mortality rate, including all cases dying from rabies within 12 months of the cessation of treatment.

Work of van Stockum

van Stockum (1935, 1938) attempted to assess the value of prophylactics by comparing the percentage of cases with short incubations to the total number of cases She based her conclusions on a statistical analysis of deaths, and thus considered only those actually at risk She concluded that a vaccine from monkey brain was the most effective Greenwood (1945–6) examined her arguments, and concluded that there was no justification for her condemnation of methods other than her own

League of Nations Reviews

From the time of the First International Conference on Rabies in Paris (1927) till his death in 1943, Colonel A. G McKendrick analyzed the statistics submitted to the League of Nations in a series of Statistical Reviews (McKendrick, 1930, 1932 *a*, *b*, 1933, 1934, 1935, 1937, 1938, 1940). He analyzed the figures relating to 1,061,707 treated persons, of these persons 3,540 died of rabies, giving a mortality of 0.33 per cent.

After Colonel McKendrick's death, returns were analyzed by the scientific

Far East, communication may be very slow, and at any rate distances great. In such places decentralization affords the best method of securing rapid and efficient treatment for all persons bitten. In these circumstances phenolized virus is undoubtedly the method of choice. All that is required is a suitable consulting room equipped with an ice-chest and facilities for administering injections in an aseptic fashion. The physician-in-charge does not require to be a whole-time expert, although he must be trained in antirabies methods and able to recognize the first signs of neuroparalytic complications.

With regard to decentralization, Cunningham and Malone (1930) emphasized the important point that in India, if there is no decentralization, only a fraction of persons at risk is actually treated They made the following recommendations

1. Decentralization of treatment should be carried out for Classes 1 and 2 (Hempt).
2. Classes 3 and 4 should be treated at a central institute.
3. Success depends on an accurate classification of risk, and Classes 3 and 4 must be dispatched speedily to Kasauli.

For further information on the work of the Kasauli Institute and its out-stations, see Webster (1944).

DETERMINATION OF THE RELATIVE EFFICACY OF VARIOUS METHODS

It will be evident that there is a large number of different methods of antirabies treatment in use throughout the world, and the question arises How do they compare with one another in immunizing efficiency?

This problem can be approached from two aspects. the experimental and the statistical. With regard to the first, the usual method of testing the potency of vaccines is to immunize animals, and later subject them to a test injection of virus

This question has been fully discussed in the previous chapter. It will be recalled that experimental work has amply demonstrated that animals can be immunized with potent strains, so that they withstand a subsequent test dose of active virus Live and chemical vaccines (phenol-, formol-, chloroform-, ether-, treated) have all been shown to be effective under these conditions. The most effective are known to be irradiated brain tissue vaccines or tissue cultures. When antirabies vaccines are given to animals after infection, it has been found difficult to secure protection, although very large doses have some effect Irradiated vaccines have proved very much more effective As far as experimental work goes, it suggests that the commonly used types of vaccine are not particularly potent immunizing agents, and not as efficient as the newer irradiated vaccines No striking superiority of any of the older types of vaccine has been definitely demonstrated by experimental studies

An elaboration of the experimental approach is to immunize human cases with various types of vaccine, if possible, the cases to be treated with each type of vaccine should be chosen strictly alternately For instance, such experiments were carried out by Cunningham, Malone, and Craighead (1933) using carbolized, Alivisatos's, and ether-carbolized vaccines Another method of assessing the relative efficacy of various treatments will now be described, viz, the study of mortality rates in treated cases

MORTALITY FROM RABIES IN TREATED CASES

Despite the application of antirabies treatment, a number of patients, none the less, dies of rabies

(*b*) Licks on intact mucosae of mouth, nose, eyes, anus, or genito-urinary tract.
(*c*) Bites or scratches raising epidermis but not drawing blood, except on head, neck, or fingers

Class 2. (*a*) Licks on fresh cuts on fingers
(*b*) All bites and scratches on fingers which are not lacerated, not over ¼" long and have not penetrated true skin
(*c*) Bites and scratches on all parts of the body except head, face, or fingers, definitely drawing blood, but excluding bites 5 or more in number or in which extensive laceration has occurred.
Any case in this category 14 days or more late in commencing treatment will be treated as for Class 3.

Class 3. (*a*) Licks on fresh cuts or abrasions on head or neck.
(*b*) All bites or scratches on head and neck.
(*c*) Bites or scratches on fingers which are lacerated, over ¼" long or have *penetrated true skin*
(*d*) All bites penetrating the true skin and definitely drawing blood when there are 5 or more in number
(*e*) All bites on any part of body causing extensive laceration.
(*f*) All jackal and wolf bites and licks

(*c*) *The position of the bite.* McKendrick (1940) found that the mortalities for Europeans and non-Europeans according to the position of the bite were as follows

	Europeans	*Non-Europeans*
Head	1.29	3.41
Arm	0.15	0.53
Trunk	0.02	0.22
Leg	0.05	0.27

The mortalities for Europeans bitten on the head, arm, trunk, and leg were in the ratio of 100·12 2 4, while for non-Europeans the corresponding ratios were 100.15:6 8.

(*d*) *The intervention of clothing* McKendrick (1938) reported that in the case of Europeans bitten on the bare skin, the mortality was 8 times that in those bitten through clothing; for non-Europeans it was 3 3 times as great for those bitten on the bare skin as through clothing.

(*e*) *Delay in commencement of treatment.* McKendrick (1940) found in the

cent. died

The detailed figures are

Time of Arrival	*Mortality* Non-European	European	*Ratio of Mortality* Non-European/European
0–4 days	0.47	0.19	2.5
5–7 days	0.47	0.10	4.7
8–14 days	0.50	0.07	7.1
over 14 days	1.08	0.12	9

It is probable that these figures do not point to a true racial susceptibility. It is more likely that the non-European arriving late is more at risk than the European.

There has been a number of recent reports referring to cases dying of rabies despite very prompt treatment, starting even within a matter of hours (Denison

ma-
all

staff of the Health Section of the League of Nations. This 10th (and final) review, presented in a paper by Greenwood (1945–6), referred chiefly to 1938 and 1939 with some figures up to 1944. Including figures supplied by the U.S.S.R., the 10 reviews referred to 1,670,848 treated persons.

Tables 34 and 35 give the figures relating to mortality in the 10th review and all 10 reviews. The mortality from rabies in treated European persons over the period of all the reviews is seen to be 0.15 per cent., and in non-Europeans 0.52 per cent.

Factors Influencing Mortality Rates

Mortality rates such as have been quoted in Tables 34 and 35 are dependent upon a number of factors; for the following information we are mainly indebted to the work of McKendrick and his coworkers (McKendrick, 1928, 1940, McKendrick and Fox, 1917; Harvey and McKendrick, 1923, 1930). We shall quote chiefly from McKendrick's last (9th) review, the figures for the 10th review being substantially similar.

(*a*) *The race*. This is by far the most important factor determining mortality from antirabies treatment. For the period covered by McKendrick's 9 reviews the mortality among non-Europeans was no less than 3.7 times as great as among Europeans (Europeans 0.15 per cent., non-Europeans 0.56 per cent.). Rabies mortality figures are, accordingly, usually given separately for Europeans and non-Europeans. In the 9th review, 53.5 per cent. of treated persons were Europeans, and for the period of all 9 reviews the percentage was 53.7 per cent.

It is unlikely that there is, however, a true racial susceptibility of the non-European. It is probable that as Europeans are usually bitten through clothing they are less at risk than non-Europeans. Also the statistics for Europeans are greatly "diluted" by the inclusion of large numbers who take treatment as a precaution, without being at risk.

(*b*) *The severity of the bite* influences the mortality as follows. In Europeans the mortality in deep bites was 6.5 times as great as in more superficial ones, whereas, in non-Europeans deep bites were only 3.3 times as serious as superficial (McKendrick, 1940). McKendrick (1928) gave the following information with regard to the mortality following bites, according to the number of tooth marks thus, after bites with only 1 mark the mortality was 0.31 per cent., with 3 marks 0.88 per cent., with 6 marks 2.22 per cent., and with 12 marks 4.76 per cent.

Hempt's (1925) classification has proved a useful method of estimating the severity of the bite and the degree of risk to which the person is exposed (see e.g., Cunningham and Malone 1930). *Four classes are recognized*

Class 1. These patients have not been bitten, but the saliva of a rabid, or suspectedly rabid, animal has gained access to cuts.

Class 2. These patients show superficial bites on the trunk or limbs (excluding fingers).

Class 3. (*a*) This class includes all superficial bites on the fingers.
(*b*) Here there are superficial bites on all parts of the body (not the head and neck), more extensive than in Class 2.
(*c*) In this class the patient suffers from deep bites in a few areas (not the head and neck).

Class 4. (*a*) Here there are deep bites all over the body.
(*b*) Any bite or scratch on the head and neck.

That this classification affords a fair estimate of the risk to which a bitten person is exposed is shown by the following figures of Cunningham and Malone (1930). with Class 1 bites, the mortality was nil, with Class 2, 0.31 per cent., Class 3, 1.04 per cent., and with Class 4 the mortality was 7.93 per cent.

Indian classification. The following classification was adopted in India in 1939 (Webster, 1944).

Class 1. (*a*) Licks, including direct contact with saliva, on fresh cuts except on head, neck, or fingers.

3. Cauterization is obviously of some value in protecting against rabies.

4. Rabies virus is an effective antigen, but certain strains have shown very feeble immunizing potency. Animals can be actively immunized especially with live, phenolized, chloroformized, and irradiated vaccines, so as to withstand a subsequent intracranial test dose of virus.

5. It is difficult to prevent rabies developing by injections of vaccine given after infection, but this has been secured by certain workers in small laboratory animals.

6. Various phenolized vaccines tested in the U. S. A. have been found to be almost inert in inducing active immunity. In the past, a considerable number of persons must have been treated with vaccines of very low or negligible immunizing activity.

7. Vaccines prepared by exposure of brain tissue to ultraviolet light have been shown to be the most effective immunizing agents in animals. These vaccines have not been used in man.

8. The mode of operation of antirabies treatment is unknown, but it may depend on the interference phenomenon.

9. Large numbers of persons treated with antirabies vaccines are not at risk. Owing to difficulties of "follow-up," numerous deaths from rabies after treatment are not so recorded. The published figures for mortality from rabies in treated persons are, therefore, fallaciously low.

10. It is only rarely possible to tell if any given treated person has actually been infected with rabies virus or not. Even if the biting animal is proved rabid, this does not mean that an infective dose of rabies virus has necessarily been deposited in the wound.

11. McKendrick's reviews do not point to the superiority of any one type of commonly used vaccine.

12. Mortality from rabies after treatment, as in the case of untreated persons, depends on the quantity of virus inoculated, and the amount of trauma caused.

We know that antirabies treatment can act in small laboratory animals, even when administered after infection. There is, therefore, every justification for expecting some benefit from the treatment in man.

Surprisingly few papers have been published of recent years bearing on the value of antirabies treatment in man, where adequate control untreated groups have been observed.

Cornwall (1923) stated that the only way to evaluate treatment is to take those instances where a presumably rabid dog has bitten several persons, some of whom are treated and some not. Both groups must be observed for several months. He gave these figures from S. India:

Total number of persons observed	2174
Number treated	812
Deaths among treated	2.9 per cent
Number untreated	1362
Deaths among untreated	6.2 per cent.

He therefore concluded that treatment saved one out of every two who would otherwise have developed hydrophobia.

Yu (1941) in China, observed 201 patients fully vaccinated after dog bites, of whom 9 died, giving a mortality of 4.5 per cent. Two of these cases started treatment late. In 182 unvaccinated persons there were 18 cases of rabies, giving a mortality of 10 per cent.

Taking both these sets of figures at their face value, it appears that antirabies treatment halves the mortality. In an attempt to obtain further information, we have communicated with M. Remlinger who kindly wrote at length, along the following lines (1946*a*):

methods of treatment, for persons bitten by dogs was 0.30 per cent., cats 0.04 per cent., wolves 8.70 per cent., jackals 1.48 per cent. For the same period, only 1 person died from the bite of a soliped (actually an ass). For the whole reviews, of 33,626 persons bitten by solipeds and ruminants, only 3 cases developed rabies. Of 11,134 persons who came in contact with human rabies, only 1 contracted the disease.

(g) *Evidence of rabies in biting animal.* The biting animal can be classified under various headings, according to the degree of certainty with which it is thought to be rabid. Category A—animal is proved rabid by experimental means, B—animal is certified rabid by medical or veterinary physician; C—animal is only suspectedly rabid, D—animal is not suspected of being rabid. It is evident that the persons bitten by animals in Categories A, B, and C will show a higher mortality than those in D. For example, McKendrick (1940) reported a mortality of 0.30 per cent. after bites from animals in Category A as compared with 0.009 per cent. in Category D, 0.18 per cent. in Category B, and 0.29 per cent. in Category C. The important dividing line is thus between Category D and the others.

Conclusions

McKendrick (1940) in summarizing his examination of statistics relating to approximately a million persons found little evidence of superiority of one method over another. He thought it unlikely that a difference in efficiency of more than some 25 per cent. existed between any two of the methods. There was, however, *the possible exception that method 9 (a) employing dilutions with, in severe cases, the addition of Alivisatos's vaccine might be superior to the others.*

Greenwood (1945–6), in his analysis of the whole 10 reviews of the League of Nations, came to the conclusion that there was no evidence enabling him to decide that any one method has a definite superiority over the others. He found that it is impossible to overcome the difficulties of comparing groups of treated persons in whom the degree of risk is unknown. It does not appear that any further compilation of data can throw any more light on the relative efficacy of the different treatments.

Before leaving this question of the relative efficacy of various methods of treatment, we would like to impress one point upon the reader: although the above-quoted observations may not suggest the superiority of any one method, there is another factor to be considered. That is, after any type of vaccine the patient may develop a serious accident, and these are much less common with some types than others. For instance, McKendrick (1940) found that the incidence of accidents was less than 1: 8,000 in those treated by killed phenol, killed ether, and heated vaccines, 1: 3,398 in those treated by living cords, 1: 3,194 by dilutions, and approximately 1: 2,000 for those treated by methods 9 (*a*) or (*c*). It is this lower incidence of fatal accidents following killed vaccines, rather than any superiority in their immunizing capacities, that has prompted so many to abandon living vaccines.

THE VALUE OF ANTIRABIES TREATMENT IN GENERAL

From what has been said above, it will be evident that there is a large number of factors complicating the evaluation of antirabies treatment. This question has been discussed by one of us elsewhere, and the following remarks are largely quoted from this paper (Rhodes, 1946).

The main factors to be considered are:

1. The mortality from rabies in untreated persons bitten by rabid animals is on the average not more than 15 per cent., and may be as low as 5 per cent. The mortality figure varies directly with the amount of virus inoculated in the wound and the degree of trauma.
2. Strains of street virus probably vary in virulence and possibly in antigenic structure.

We question, however, whether in many institutes nearly as many as 500/1000 treated persons are actually at risk following dog bites. If only 100 are at risk, and the mortality in untreated cases is 10 per cent, then only 10 persons would die in our group of 1000 without any treatment.

One cannot, therefore, put forward a strong case in support of antirabies treatment by arguing on general lines, rather than from figures of more or less controlled series such as that of Cornwall.

When we come to discuss the position in the much severer wolf bite, there seems to be more satisfactory evidence of the value of treatment. The mortality from untreated wolf bites, due to rabies, is generally believed to be not less than 60 per cent, and it is said to be very rare for wolves to bite unless rabid. Accordingly, of 1000 persons bitten by wolves practically all may be considered to be genuinely at risk—so that not less than 500–600 of the 1000 should die from rabies if untreated. The mortality from rabies in treated cases of wolf bite is not more than 15 per cent. (Remlinger, 1946 *a*). If one accepts the high mortality in untreated cases, and the statement that wolves seldom bite unless rabid, it would appear that antirabies treatment is of definite value.

We have also received a personal communication from Professor Lépine of the Pasteur Institute of Paris (1946), who firmly believes in the efficiency of antirabies treatment, and sees no reason for throwing suspicion on the value of the method.

In conclusion, on the evidence at present available, we regard antirabies treatment as being of some value in the prevention of rabies, although it may not necessarily act by the process of active immunization. There is no justification for withholding treatment from persons thought to be at risk. The treatment as at present employed could probably be considerably improved, partly by the greater use of immune serum, and partly by the greater use of more potent vaccines.

There is a wide field for further research, particularly into the mechanism involved in the acquisition of the immune state. It is most desirable that every effort should be made to secure statistical evidence concerning the value of antirabies treatment, by comparing the incidence of the disease in treated persons, and in those exposed to the same risk who for some reason have not taken treatment.

Let us consider 1000 persons treated in an antirabies institute for dog bites. Probably only 500 of these have been bitten by rabid dogs. As the death rate in such persons may be 15 per cent. we may expect 75 of the 1000 persons to die of rabies if untreated The mortality in treated persons is, however, only about 5 per 1000. By this line of argument it appears as if antirabies treatment saves 70 persons per 1000 treated.

TABLE 34[1] **(from Greenwood, 1945–6)**

	Tenth Review				*All Ten Reviews*			
		Deaths				*Deaths*		
	Number treated	*Observed*	*Calculated*	*Mortality %*	*Number treated*	*Observed*	*Calculated*	*Mortality %*
Europeans.								
1. Cords	14,933	12	22	0·08	79,958	107	122	0·13
2 Dilutions	10,734	11	16	0·10	84,098	136	128	0·16
3 Killed phenol	18,975	48	28	0·25	100,530	183	154	0·18
4 Live phenol	2,691	2	4	0·07	9,697	8	15	0·08
5. Fermi's vaccine	8,667	2	13	0·02	26,390	25	40	0·09
6 Fermi's s-v	—	—	—	—	390	0	1	0·60
7 Heated	22,334	39	32	0·17	163,293	297	249	0·18
8. Killed ether	28,556	47	41	0·16	119,475	159	182	0·13
9 Mixed (a)	5,807	3	8	0·05	63,034	68	96	0·11
(b)	3,116	4	5	0·13	10,423	18	16	0·17
(c)	—	—	—	—	6,646	8	10	0·12
10 Yatren	—	—	—	—	5,384	13	8	0·24
TOTAL	115,813	168	169	0·15	669,318	1,022	1,021	0·15
	$\chi^2 = 36·4$ P = 0·00005				$\chi^2 = 41·4$ P = 0·00003			
Non-Europeans								
1. Cords	17,580	55	53	0·31	98,532	479	508	0·49
2. Dilutions	—	—	—	—	1,777	16	9	0·90
3 Killed phenol	63,631	209	193	0·33	447,586	2,403	2,308	0·54
4 Fermi's vaccine	14,802	27	45	0·18	24,403	53	126	0·22
TOTAL	96,013	291	291	0·30	572,298	2,951	2,951	0·52
	$\chi^2 = 8·6$ P = 0·014				$\chi^2 = 53·3$ P < 0·00001			

TABLE 35[1] **(from Greenwood, 1945–6)**

	Tenth Review				*All Ten Reviews*			
		Deaths				*Deaths*		
	Number treated	*Observed*	*Calculated*	*Mortality %*	*Number treated*	*Observed*	*Calculated*	*Mortality %*
Europeans								
(a) Killed	47,531	95	69	0·20	220,005	342	336	0·16
(b) Live	37,281	32	54	0·09	247,210	337	377	0·14
(c) Heated	22,334	39	32	0·17	163,293	297	249	0·18
(d) Other	8,667	2	13	0·02	38,810	46	59	0·12
TOTAL	115,813	168	168	0·15	669,318	1,022	1,021	0·15
	$\chi^2 = 29·6$ P < 0·00001				$\chi^2 = 16·5$ P = 0·00009			
Non-Europeans								
(a) Killed	63,631	209	193	0·33	447,586	2,403	2,308	0·54
(b) Live	17,580	55	53	0·31	100,309	495	517	0·49
(d) Other	14,802	27	45	0·18	24,403	53	126	0·22
TOTAL	96,013	291	291	0·30	572,298	2,951	2,951	0·52
	$\chi^2 = 8·6$ P = 0·014				$\chi^2 = 47·1$ P < 0·00001			

[1] See footnote to Table 36

2. They may suffer from a neuroparalytic accident, which may prove fatal. The exact etiology of this condition is still uncertain.

1. *Rage De Laboratoire*

The term *rage de laboratoire* was introduced in the early days and applied to certain cases which died after antirabies treatment. The term carried the insinuation that the treated person had been infected and died from the *virus fixe* in the injection that was supposed to save his life. There is no doubt that genuine cases of *rage de laboratoire* have occurred. In these, the patient has died following antirabies treatment with live vaccine, and it has proved possible to isolate *virus fixe* from the central nervous system (Bareggi, 1889, Athias and França, 1909–10, França, 1910, Remlinger, 1935 *a*, Proca and Bobes, 1940). Considerable interest has always been taken in this condition in France, and Remlinger (1935 *a*, *b*) has published a very complete review of the subject.

In many of these cases, the person has been bitten by a nonrabid animal, or may not even have come in contact with rabies at all (e g , Bareggi, Athias and França). This has led some persons to claim that all cases of neuroparalytic accident (*vide infra*) are due to *virus fixe*, i e , are cases of *rage de laboratoire* Such a theory cannot be generally accepted today, as neuroparalytic accidents have also followed the use of killed vaccine and, in animals, of normal nervous tissue.

Clinical features.

The following account of the symptoms of *rage de laboratoire* is taken largely from Remlinger's authoritative review The disease usually begins immediately after a course of antirabies treatment, or up to 40 days later. At the onset the patient is febrile and weak The lower limbs become completely flaccid, and there is retention of urine and feces, the paralysis spreads up to produce dyspnea, dysphagia, dysarthria, and cardiac irregularities There are no symptoms of hydrophobia.

Incidence.

The incidence of *rage de laboratoire* is distinctly low, probably not much over 30 cases have been recorded, but Remlinger (1935 *a*) believes that this figure gives a wrong conception of the true position. He thinks that many cases are missed owing to an inadequate "follow up" system, others are misdiagnosed or miscertified, while in others no autopsy or animal inoculation test is carried out.

Etiology.

To isolate the rabies virus from suspected cases of *rage de laboratoire*, a suspension of the bulb should be injected into a rabbit Supposing the animal dies from rabies these questions arise First, is the virus a street or a fixed strain? Second, supposing it is a fixed strain, what is the significance of its presence in the bulb?

Differentiation of street and fixed strains

Often the differentiation may not be a difficult matter. The paralytic symptoms in the inoculated rabbit may come on quickly, and there may be an absence of Negri bodies, both suggesting the [illegible]
toms in the rabbit may come o[illegible]
Negri bodies, thus indicating that [illegible]
but with an ordinary rabies due t[illegible]
ing between strains of *virus renfo*[illegible]
for, as they are seldom present ir[illegible]
may show the strain to be possess[illegible]
however, *virus fixe* is isolated, what does this mean?

CHAPTER LXXIX

REACTIONS AFTER HUMAN ANTIRABIES TREATMENT

A. BENIGN REACTIONS

General Systemic Reactions after Antirabies Treatment

VARIOUS minor disorders may develop during or after a course of antirabies treatment such as fever, headache, giddiness, insomnia, palpitations, and diarrhea. Cunningham and Malone (1930) recorded a case of generalized angioneurotic edema.

In a case described by Palmer (1941) there was constipation and lack of consciousness of the act of defecation.

A number of cases of sudden shock-like collapse or fit has been recorded, usually coming on towards the end of a course of treatment, it appears that these reactions are due to sensitization to protein contained in the vaccine, are commoner in persons giving a history of allergy, and can be largely avoided if smaller doses are given, desensitization can also be attempted (Dodero, 1938 *c*, Bernard, 1939, Horack, 1939, Lépine, 1939, Dorfman, 1940, Noury, 1940, Palawandow, 1940) It appears that this shock does not occur if the vaccine is prepared from dog or sheep brain instead of rabbit brain (Raynal and Lieou, 1946) A shock-like syndrome has been produced in rabbits by repeated injections of phenolized brain (Raynal and Lieou, 1940 *b*)

Albuminuria is said to occur in 10 per cent of cases (Auffret and Dodero, 1940 *a*).

Local Reactions after Antirabies Treatment

Local reactions of the skin after injection of antirabies vaccine have been recorded by various authors (*Cunningham and Malone*, 1930, *Lépine and Cruveilhier*, 1935 *a*, Cruveilhier *et al*, 1939) and by Remlinger (1927 *c*)

1 *Erythematous patches* This lesion usually develops about a week to 10 days after the beginning of antirabies treatment, when a few hours after an injection of vaccine an erythematous patch appears, which may measure some centimeters in diameter *This patch usually fades away after about 6 to 8 hours, only* to reappear after every subsequent injection The erythema usually becomes less marked with later injections, and after 15 to 16 may only be very slight. This type of reaction is more severe after the use of etherized vaccine than carbolized

2 *Urticarial reactions and rashes* These were found to occur in 16 per cent. of cases treated with etherized or carbolized vaccines (Cunningham and Malone).

3 *Abscesses* These develop only rarely and may be acute or chronic, they are due to an error in aseptic technique

It has been stated that if these local reactions occur in the first course of antirabies treatment, they may be much less marked, or even absent, in a second course (Kaktine, 1931) Cunningham and Malone, however, found that erythematous patches were more severe in those undergoing antirabies treatment for the second time It has been suggested that a functional liver disturbance is responsible for these reactions (Cruveilhier *et al*, 1939)

B. SEVERE AND FATAL REACTIONS

Patients may suffer from serious, often fatal, illnesses of two types after antirabies treatment, fortunately these are extremely rare

1 Persons may die of *rage de laboratoire* That is to say, the virus in the vaccine proves pathogenic, after death, *virus fixe* may be isolated from the brain

that the presence of this virus is not incidental, and that it is the definite etiological agent of *rage de laboratoire*.

We would emphasize that although *rage de laboratoire* is a very rare condition, it is a most tragic one, for it cannot occur if killed vaccines are used. It is probably largely for this reason that so many present-day workers prefer to use killed vaccines, in preference to living vaccines which have no greater immunizing powers.

2. Neuroparalytic Accidents

This term is applied to cases which develop certain neurological manifestations after undergoing a course of antirabies treatment. Similar accidents may also occur in animals (*vide infra*) The syndrome should be sharply delimited from neurological disorders produced by street virus (e g, the landriform type of rabies), and by fixed virus (*rage de laboratoire*), for neuroparalytic accidents are quite distinct from these. Cases of neuroparalytic accident do not show hydrophobic symptoms, pathologically they differ from rabies, no Negri bodies are found, and injection of brain yields no virus.

The condition has attracted much attention in the literature, especially the French. Remlinger (1905 *a*) published one of the earliest papers on the subject, in which he reviewed some 40 cases (see also 1908 *a*, 1927 *a*). Fielder (1916) reviewed some 13 cases. In reporting the proceedings of the First Conference on Rabies, Remlinger published another authoritative review on the question (1927 *b*). The slightly later paper of Stuart and Krikorian (1928) should also be consulted. Remlinger (1932) reviewed 66 cases occurring after his 1927 paper; later (1937 *a*) he reviewed 202 cases since 1932 Other papers of interest may be mentioned (Paulian *et al.*, 1939; Jones and Ellinger, 1940–1, Dang, 1941, Thomas, 1945, Sellers, 1947).

Clinical features.

Factors predisposing to neuroparalytic accidents. It has been recognized for a considerable time that certain persons are more prone to develop neuroparalytic accidents than others (see, e g, Babès, 1909 *a*, Report of Rabies Conference, see p. 800, Remlinger, 1927 *b*). These accidents are more frequent in brain workers than manual laborers, the patients are often nervous and "highly strung" Alcohol, syphilis, chill, fatigue, and traumata are also thought to have some predisposing influence

Incubation period The accident usually develops between the 13th and 15th day of antirabies treatment.

Symptomatology. Neuroparalytic accidents usually assume one of three forms (*a*) the Landry type, (*b*) the dorsolumbar paralytic type, and (*c*) a neuritic type often characterized by facial paralysis. At the Rabies Conference some 243 cases were reported, mostly falling into one or other of these groups Gordon (1935) has [illegible]

[illegible] ccident have been [illegible]) or (*c*) (Rendu, [illegible], 1932, Stuart and Krikorian, 1930, 1933, Ch'en and Ch'eng, 1936; Idei, 1937). Symptoms usually arise towards the end, or shortly after the completion, of the course of antirabies treatment. [illegible]

[illegible] stated to be about 30 per cent, and in the remainder recovery usually occurs rapidly. At the Rabies Conference 39 out of 243 reported cases fell into this group

(*b*) *The dorsolumbar type.* This form is less severe than the Landry type, and is the commonest type of neuroparalytic accident. The clinical features are ex-

The significance of virus fixe *in the central nervous system*

Does the presence of *virus fixe* in the human central nervous system mean that it is necessarily pathogenic? May not *virus fixe* be present in the brain of all cases treated by living or attenuated vaccines?

Quast (1926) claimed to have isolated a strain with properties suggestive of *virus fixe* from the bulb of a person subjected to antirabies treatment, who presented no clinical signs of rabic infection. Further, he treated dogs with rabies vaccine and demonstrated virus in the brain. Similar results were obtained in injected animals by Isabolinski and Zeitlina (1929). These observations suggested that the presence of *virus fixe* in the central nervous system of treated persons might be quite usual. Were these observations confirmed, it would cast serious doubt on there being such a condition as *rage de laboratoire* at all, the presence of *virus fixe* in the central nervous system being evidently a normal occurrence

However, much experimental work has been carried out in which these findings have not been substantiated. Thus cats, dogs, rabbits, and guinea-pigs were immunized by the desiccated cord method and with living *virus fixe*. When the animals were killed after some lapse of time no virus could be isolated from the central nervous system (Remlinger, 1910, 1927 *b*, Remlinger and Bailly, 1927 *a*, 1928 *b*, 1931 *a*; Schnürer and David, 1927; Burnet, 1928; Nicolau and Kopciowska, 1932 *b*, and see Report of Rabies Conference). No virus could be isolated by cataphoresis (Nicolau, Viala, and Kopciowska, 1930; Cruveilhier, Nicolau, and Kopciowska, 1935). Not even when the vaccines were prepared from the active Chisinau strain could virus be demonstrated in the central nervous system of injected animals (Remlinger, Palmowitch, and Bailly, 1931 *a*).

As Quast's observations have not been confirmed by the bulk of experimentalists, it is now generally agreed that *virus fixe* does not occur in the central nervous system without having some pathogenic significance. The next point to consider is, what evidence is there that *virus fixe* is sufficiently pathogenic for man to cause a fatal infection?

Virulence of virus fixe *for man*

A number of observations is on record which suggest the innocuity of *virus fixe* for man. Various persons have injected human beings with living or slightly attentuated fixed virus, subcutaneously and even intravenously, with no harmful results (Wissokowicz, 1902, Nitsch, 1904, Remlinger, 1935 *a*). Further, as Remlinger points out, although rabies has frequently been contracted by persons carrying out a postmortem on a naturally infected animal, the same accident has not befallen those working with infected passage animals in the laboratory.

Although tacitly assumed on the strength of the above experiments, the avirulence of *virus fixe* for man is not absolute Thus, a number of cases of rabies has followed the use of attenuated or virulent vaccines, *virus fixe* could be isolated from the central nervous system In these cases the possibility of naturally acquired rabies was ruled out as, although the person may have been bitten, the animal was proved to be healthy (see, e g, Ferran, 1888, Bareggi, 1889, França, 1910).

Other factors influencing the etiology.

Remlinger (1935 *a*) has stated that the method and site of injecting the vaccine is of importance Thus, if many nerve endings are traumatized, the virus may be able to ascend in the nerves more readily and cause rabic infection of the central nervous system He has also noted a tendency for the more intellectual classes to be affected, and for the cases to occur in small groups of two or three

Conclusions.

We may conclude that in certain, fortunately very rare, fatal cases following antirabies treatment with live vaccine, *virus fixe* may be isolated from the brain,

Of the 2 cases that complicated antirabies treatment, one had been bitten by a healthy camel, and the other had not been exposed to rabies at all

Histologically, all these cases presented marked evidences of widespread involvement of the ganglion cells in the cord The primary changes were nuclear, and the following types of reaction might occur. (*a*) the clear basophilic type of nucleus with somewhat coarse chromatin, (*b*) the dark basophilic type which was deeply stained, with prominent reticulum and chromatin bodies, and with or without a nucleolus, (*c*) the dark basophilic homogeneous type where the deeply stained nucleus was structureless All these types of nuclear change (but especially "*a*") might precede karyolysis. There was no demyelination or perivascular cuffing, and apart from some edema, the remarkable nerve cell changes were the only features

Relationship to other types of "postinfective" encephalomyelitis. Perdrau (1928), in an authoritative article on encephalomyelitis in general, concludes that the same pathological changes occur in true postvaccinal encephalitis, measles encephalitis, and rabies neuroparalytic accidents. Perivascular demyelination has, however, only rarely been found in cases of neuroparalytic accident. Undoubtedly when this occurs the picture closely resembles that of true postvaccinal encephalitis, but many reported cases have not resembled this condition. The etiology of postvaccinal encephalitis and related disorders is still *sub judice*, this subject is discussed elsewhere (see Ch XXXV).

Epidemiology and incidence.

Remlinger (1927 *b*) has given much information on this question (see also Stuart and Krikorian, 1928) Thus, children suffer much less commonly from neuroparalytic accidents than adults, the majority of cases being over 15 years of age. The sexes are equally prone to develop neuroparalytic accidents, although men are more predisposed to bites than women Intellectuals are more prone to attack than laborers, and Europeans may be more so than Eastern races, although this is not a general rule.

There appears also to be a distribution of neuroparalytic accidents according to the institute One institute using the same method as another institute may have a high incidence of neuroparalytic accidents, while the other escapes. Certain institutes, for example, the Pasteur Institute in Paris, seldom seem to have cases of neuroparalytic accident, although at others they are quite frequent.

Incidence of neuroparalytic accidents Remlinger (1927 *b*) is of the opinion that many minor cases of neuroparalytic accident fail to be reported and that the true incidence is higher than any published tables would lead one to suppose. He gives the following reasons why such understatements should occur (*a*) an imperfect "follow up" of treated cases, (*b*) other causes may be blamed for the paralysis, such as syphilis, alcohol, and influenza, (*c*) cases may definitely be concealed by official reporters.

We quote (Table 36) the figures given by Greenwood (1945–6) for the incidence of neuroparalytic accidents for the period since the Rabies Conference in 1927, according to the method of treatment

The incidence of neuroparalytic accidents in the main methods of treatment will now be discussed, referring both to Greenwood's figures and to the earlier reports of the Rabies Conference (Remlinger, 1927 *b*)

(*a*) The desiccated cord method gives a high percentage of neuroparalytic accidents (mainly nonfatal), although in the Pasteur Institute in Paris from 1911 to 1927, among 13,450 treated cases there was not one of this complication A week's treatment of cords in glycerol tends to reduce the incidence of neuroparalytic accidents

(*b*) Hoegyes's dilution method also gives a high incidence of neuroparalytic accidents, which are mainly fatal At the Rabies Conference it transpired that, although in Budapest the rate was low (0 39 per cent.), at Breslau it was 1 05 per

plicable by the presence of a dorsolumbar myelitis (Remlinger, 1905 *a*, 1927 *b*; Tartakoff and Vibber, 1932, Stuart and Krikorian, 1933; Marinesco and Draganesco, 1938). The patient may be slightly febrile, and feels weak, the lower limbs become paralyzed, usually with diminished sensation, and sphincter disturbances may occur. Although transient weakness may involve the upper extremities, there are never any of the severer bulbar symptoms. The fatality rate is not over 5 per cent. (Remlinger), the majority of cases recovering quickly.

At the Rabies Conference there were 68 out of 243 cases of paraplegia with sphincter disturbances, 33 cases of paresis of the lower limbs, with urinary retention, and 21 cases without retention.

(*c*) *The neuritic type.* The patient may be pyrexial, and usually shows a temporary paralysis of the facial, oculomotor, glossopharyngeal, or vagus nerves. At the Rabies Conference 58 out of 243 cases of facial paralysis were reported, and a few cases of paralysis of limbs and other cranial nerves. A case of double papilledema after antirabies treatment with carbolized vaccine has been recorded (Cormack and Anderson, 1933) also another case of optic neuritis (Koenigsfeld, 1945).

(*d*) *Other syndromes.* Gordon (1935) described cases with an abrupt onset, pyrexia, headache, and pains in the back, stiffness of the neck and Kernig's sign, increased reflexes, facial paralysis, and lymphocytosis in the cerebrospinal fluid.

The period of incapacity from neuroparalytic accident usually lasts 5 to 20 days, but in certain cases may be very prolonged, even up to 5 months (Lépine and Cruveilhier, 1935 *a*).

Pathology.

The majority of descriptions of the pathology refers to the landriform type of accident, as the other types only rarely prove fatal (see, e.g., Babès and Mironesco, 1908, Koritschoner and Schweinburg, 1925, Bassoe and Grinker, 1930, Getzowa, Stuart, and Krikorian, 1933, Stuart and Krikorian, 1933, Ch'en and Ch'eng, 1936).

General changes. It is somewhat difficult to give a systematic account of the pathology, as there is no change uniformly present in all cases. The following is a general account of the changes that frequently occur in the cord, the brain usually showing little beyond some edema and congestion.

The cord is soft, especially in the lower dorsal and lumbar areas, where the gray and white matters may be indistinguishable. Microscopically, there is much axis cylinder destruction, with considerable neuroglial proliferation. The vessels in the gray matter show perivascular cuffing with leukocytes and embryonal cells. Degeneration and satellitosis of the nerve cells may be seen. Negri bodies do not occur and the lesions are quite different from those of ordinary rabic infection. Demyelination occurs only rarely, as in Bassoe and Grinker's (1930) case, in which it was situated perivascularly.

Koritschoner and Schweinburg's (1925) cases. These authors studied 7 cases and observed a variety of pathological features. One case showed generalized hyperemia only, and another congestion and edema of the cord. The remaining 5 cases all showed evidences of myelitis, either hemorrhagic, disseminated, or transverse.

Marinesco and Draganesco's (1938) case. These authors described a fatal case of flaccid paraplegia with urinary retention. They found a necrotic myelitis, most evident from the 9th to 11th thoracic segments, in this area the majority of nerve cells was destroyed. Evidences of demyelination were found in the segments bordering on these areas. They also found lymphocytic infiltrations in certain peripheral nerves, nerve roots, and sympathetic ganglia.

Getzowa, Stuart, and Krikorian's (1933) cases. An interesting study of the pathological features of neuroparalytic accidents was made by these workers in Jerusalem, and is worthy of particular note. They studied the histopathology of 3 fatal cases of Landry's paralysis, 2 of these complicated antirabies treatment, but the other was spontaneous. All 3 cases were similar clinically and histologically.

any theory that attempts to account for the production of all cases of neuroparalytic accident.

(*a*) The accidents tend to be commoner with live or attenuated than killed vaccines.

(*b*) They have followed antirabies treatment in persons who have had no contact with the rabies virus at all (Remlinger, 1927 *b*, Getzowa, Stuart, and Krikorian, 1933, Ch'en and Ch'eng, 1936).

(*c*) They occur almost equally commonly in all classes of treated persons, whether bitten or not, and irrespective of their degree of risk by Hempt's classification (see p. 870).

(*d*) The onset of the illness bears no constant relationship to the date of the bite but rather to the date [illegible]

(*e*) In animals, paralysis may f [illegible] tissue.

(*f*) The pathological features [illegible], do not resemble those of rabies, and Negri bodies are not found

The main theories will now be discussed *seriatim*.

1. *Street virus theory*. This theory is really composed of two subgroups.

(*a*) *Modified rabies theory* That neuroparalytic accidents were caused by street virus was widely believed in the early days of Pasteur treatment (see, e g, Laveran, 1891) Any departures from the usual symptomatology of rabies were attributed to a modification induced by the treatment. The theory is scarcely held valid at the present day, thus at the time of the Rabies Conference only one institute spoke in its favor, it was, however, supported by Koch (1927). The main argument against this theory is that cases of neuroparalytic accident have been reported after exhibition of antirabies treatment in normal unbitten persons Further, the incubation period is too short, there is no hydrophobia, and the pathology of neuroparalytic accidents is not that of rabies.

(*b*) It has been suggested that neuroparalytic accidents may be caused by strains of street virus that show no cross immunity reactions with the ordinary vaccine strain of fixed virus That is to say, there is no reason why the antigen in the *virus fixe* vaccine should confer protection against an antigenically different street virus (Babès and Jonesco, 1924, Téodorasco, 1928). Because in this theory neuroparalytic accidents are held to be street virus infections, the same objections apply.

2 *Anaphylaxis theory* It has been thought that the development of neuroparalytic accidents may be due to an anaphylactic reaction occurring in the nerve tissues This theory was first put forward by Harvey and McKendrick (1907), who concluded that accidents were due to an excess of foreign nerve material, and suggested that this reacted anaphylactically That antigen-antibody [illegible], a part has more recently been suggested [illegible]

[illegible] acquired [illegible] it does prove possible to kill rab- [illegible] shock, this does not in any way resemble the ordinary neuroparalytic accident (Stuart and Krikorian, 1928). Next, human beings [illegible], a second or even further course of antirabies [illegible] show no special [illegible] and Bailly, 19 [illegible] had previously [illegible] courses of antirabies treatment in anima[illegible] any particular liability to [illegible]

3 *Ferment theory* Earlier [illegible]

TABLE 36[1] (from Greenwood, 1945–6)

SHOWING THE OCCURRENCE OF PARALYTIC ACCIDENTS AFTER VARIOUS METHODS OF TREATMENT (OVER THE PERIOD COVERED BY ALL TEN REVIEWS)

		All Ten Reviews				Percentage	
	Number Treated	Accidents	Proportion 1 in	Percentage	Deaths	Fatal	Non-Fatal
1. Cords	192,402	57	3,375	0.030	8	0.004	0.025
2. Dilutions	85,875	25	3,435	0.029	18	0.021	0.008
3. Killed phenol	579,129	68	8,517	0.012	15	0.003	0.009
4. Live phenol	9,697	1	9,697	0.010	0	0	0.010
5. Fermi's vaccine	55,009	7	7,858	0.013	5	0.009	0.004
6. Fermi's s.-v.	390	0	—	0	0	0	0
7. Heated	163,293	8	20,412	0.005	2	0.001	0.004
8. Killed ether	119,476	9	13,275	0.008	4	0.003	0.004
9. Mixed (a)	63,034	39	1,616	0.062	4	0.006	0.056
(b)	10,423	2	5,212	0.019	0	0	0.019
(c)	6,646	3	2,215	0.045	0	0	0.045
10. Yatren	5,384	3	1,795	0.056	0	0	0.056
TOTAL	1,290,738	222	5,814	0.017	56	0.004	0.013

[1] Tables 34, 35, and 36 are reproduced (by kind permission of the League of Nations) from the *Quarterly Bulletin of the Health Organisation. League of Nations*, vol. 12, p. 301.

cent., and at another institute (Weltewreden) 0.28 per cent. (see also Lubinski and Prausnitz, 1926, Remlinger, 1928 *a*, 1937 *b*).

(*c*) The killed phenolized vaccines (e.g., Semple's) have always been accepted as giving a low incidence of accidents However, such may occur (see, e.g., Cunningham and Malone, 1930, Tartakoff and Vibber, 1932; Stuart and Krikorian, 1928, 1930, 1933, Ch'en and Ch'eng, 1936, Béguet and Horrenberger, 1940, Piringer, 1942).

(*d*) Both the heated and killed ether vaccines give low figures, although cases of neuroparalytic accident do occur (for heated vaccine, see Babès and Jonesco, 1924, for etherized vaccine. Patrikios and Catacouzenos, 1933).

(*e*) The remaining methods (mixed and yatren) give a high incidence of neuroparalytic accidents

Rôle of dosage in the incidence of neuroparalytic accidents. Many workers have reported an increased number of neuroparalytic accidents following an increase in the quantity of nervous tissue injected. At the Rabies Conference, Boecker (see Remlinger, 1927 *b*) stated that very intensive treatment gave rise to 0.89 per cent. of accidents, intensive to 0.32 per cent, and mild to 0.19 per cent. Further, at the Conference, of 114 cases in which the intensity of treatment was noted, 57 occurred with intensive treatment, 39 with average, and only 18 with mild

The exact amount of injected nervous tissue is not the only factor in the production of neuroparalytic accidents, the state of the inoculum is of importance also Thus, according to Cornwall and Beer (1925–6 *a*), although 38 mg. of fresh cord per kilo body weight, or 18 mg of desiccated cord, may cause paralysis in man, 94 mg of carbolized tissue per kilo has never been responsible for a neuroparalytic accident.

In conclusion, it is evident that the methods involving the use of living or attenuated virus (e.g, cords, dilutions) give a high incidence of neuroparalytic accidents, while the killed vaccines (e.g, phenolized, etherized) give a low incidence

The etiology of neuroparalytic accidents.

A large number of theories has been propounded to account for these accidents of treatment, but there is no unanimity as to the correct explanation, and it is probable that it has not yet been discovered Certain facts must be explained by

of adult rabbit and monkey brain with added adjuvants (paraffin oil and dried tubercle bacilli). Usually 3 injections were given intramuscularly at weekly intervals. Symptoms usually developed after about 3–4 weeks, and took the form of ataxia, monoplegia, hemiplegia, quadriplegia, ocular paralysis, or tremor.

The pathological changes were those of acute disseminated encephalomyelitis Perivascular areas of inflammation and demyelination were found, the white matter was chiefly involved, but there was a secondary involvement of the gray matter. The inflammatory changes were more marked than those that occur in the demye- … e reactions with rabbit

… the injection of brain … n in the perivascular tissue, giving rise to inflammation and degeneration here and in the vessel wall. The fact that lesions were produced by homologous brain may be explicable by autoimmunization, and it would be necessary to postulate a liberation of brain tissue in an antigenic form. They quote a number of instances where antibodies have been detected in human blood to normal human tissues.

Morgan (1946, 1947) made essentially similar observations independently. Two to 7 weeks after 2–3 subcutaneous injections of poliomyelitis-infected monkey spinal cord plus adjuvants, the majority of monkeys showed signs of severe involvement of the CNS. The effect was reproduced by the injection of spinal cord or white matter of brain of normal monkey, and to a lesser extent by cortical gray matter, provided adjuvants were used Peripheral nerve and kidney suspension with adjuvant proved negative. It appeared that the responsible antigen was present in white matter, tentatively not in gray matter, and not in peripheral nerve. Perhaps the essential difference lay in the nature of the myelin of the cerebral white matter as contrasted to that of peripheral nerve. It was concluded that the reaction

…

of lesions in monkeys, following injections of normal brain tissue, which resemble those found in certain human cases of neuroparalytic accident.

6 *Neurotropic virus theory* Inasmuch as certain cases of neuroparalytic accident show a histological picture similar to that of true postvaccinal encephalitis and measles encephalitis, one must explore the possibility of a similar etiology. In postvaccinal encephalitis, as is well known, it has been postulated that vaccination "lights up" some neurotropic virus lying latent in the body (Ch XXXV), and it is possible that antirabies treatment may act in the same way However, the existence of such a virus has never been demonstrated with certainty. It has also been suggested that the vaccine itself may contain a neurotropic virus capable of causing neuroparalytic accidents

7 Virus fixe *theory* Some of those who support this theory believe that *virus fixe* is sufficiently pathogenic to account for all cases of neuroparalytic accident (e g, Fielder, 1916, Busson, 1929) That a few cases of death (*rage de laboratoire*) from infection with *virus fixe* have occurred is generally admitted, but the fact that neuroparalytic accidents may develop following treatment with killed carbolized vaccines would seem to render impossible the general acceptance of *virus fixe* as the etiological agent Further, in animals, neuroparalytic accidents have followed the exhibition of normal nervous tissue.

Another serious flaw in the acceptance of this theory is the fact that *virus fixe* has only been isolated from the brain of cases dying after antirabies treatment on a few occasions However, Remlinger (1935 *a*, 1937 *a*), doubtless representing an important section of modern French opinion, says that the belief is gaining ground that this failure to isolate virus is due to the presence of an autosterilizable neuro-infection That is to say, that *virus fixe* is initially present, but is destroyed by the

were found to occur about a week after the beginning of the course, and to persist for 2 to 3 weeks after its termination. The ferments gave Abderhalden's reaction with rabbit, human, and dog brain However, they were found in the serum of a person who developed a neuroparalytic accident as well as in the sera of others who remained well.

4. *Rabies toxin theory.* The theory that neuroparalytic accidents were due to the presence of a rabies toxin in the vaccine used to be widely held, and even at the Rabies Conference there were some supporters According to this view, the neuroparalytic accident is thought to be a toxic reaction, because of its sudden onset with pyrexia and its usually rapid recovery. However, if there were such a toxin one would certainly expect very many more cases than actually occur It is difficult to accept this theory, especially as a rabies toxin has never been convincingly demonstrated

5. *Constituent of normal nervous tissue theory.* That some constituent of normal nervous tissue might be the cause of neuroparalytic accidents was suggested many years ago (see Harvey and McKendrick, 1907). It was supported at the Rabies Conference, and is still accepted by many (see, e g, Schweinburg, 1930, Loeffler and Schweinburg, 1930). It is free from the objections that affect so many of the other theories, because if a person receives antirabies treatment at all he is bound to be injected with nerve tissue Whatever is the potent constituent of the nervous tissue it must be rendered less harmful by carbolic acid

The main proof of this theory has been attempted by experimental means

(*a*) Thus, rabbits have been subjected to injections of normal homologous or heterologous brain tissue (Remlinger, 1909 *a*, 1919 *f*, *g*, 1920 *a*, *b*, Schweinburg, 1924, Koritschoner and Schweinburg, 1925, Stuart and Krikorian, 1928). In many cases the animals have presented untoward symptoms, for some have died in convulsions after 4 to 5 injections, and others have developed paralysis

(*b*) Paralysis and wasting of the limbs, eventually proving fatal, may follow injections of carbolized nervous tissue (Harvey and Acton, 1922–3), Stuart and Krikorian (1928), however, obtained no untoward reaction after large doses of carbolized brain.

(*c*) Hurst (1932) investigated the effect of injecting normal, heated, and carbolized brain suspensions (guinea-pig, sheep, human, and monkey) into rabbits He found that many animals died, some suddenly, others with marked wasting Less than one-tenth of animals showed paralysis, and microscopically there were no definite lesions The evidence was insufficient, in Hurst's opinion, to state that neuroparalytic accidents were due to normal brain tissue.

(*d*) Schwentker and Rivers (1934) also injected rabbits with brain tissue. In a proportion of cases a complete paralysis of the hind legs developed Histologically, however, there were no specific lesions and certainly no demyelination They came to conclusions similar to Hurst's Certain more positive results have, however, been recorded with monkeys

(*e*) Rivers, Sprunt, and Berry (1933) gave repeated injections of brain extracts and emulsions to monkeys Following these injections some animals developed inflammatory changes in the central nervous system with demyelination.

(*f*) Rivers and Schwentker (1935) carried out a further series of experiments on monkeys The animals were given several intramuscular injections of aqueous emulsions and alcohol-ether extracts of sterile normal rabbit brains Histological changes were produced in which myelin destruction, mainly perivascular, was prominent

(*g*) Millischer (1938) found that the cerebral tissue of healthy animals elaborates, after death, a toxic substance capable of producing paralytic accidents in rabbits

(*h*) Important observations have been made by Kabat, Wolf, and Bezer (1946, 1947), who inoculated healthy young *M rhesus* monkeys with phenolized emulsions

cidents That there may be such a syndrome as this was clearly emphasized by Hempt at the Rabies Conference (see Remlinger, 1927 *b*). He reported 2 cases bitten by a healthy dog who were not even subjected to antirabies treatment. Some 4 and 8 weeks later both patients became paralyzed As Hempt points out, if these cases had been subjected to antirabies treatment they would naturally have been classed as cases of neuroparalytic accident.

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paralysis which developed 24 days after the beginning of the injections Street virus was isolated from the central nervous system This case was almost certainly an ordinary case of landriform rabies, with the antirabies treatment as an incidental and not the etiological factor. However, were it not for the isolation of street virus it might have been labelled as a Landry type of neuroparalytic accident.

These authors reported a somewhat similar

n confused with a neuroparalytic accident,

(*c*) *Marinesco and Draganesco's (1935) case.* This case has attracted much interest and will be described rather fully Clinically the case was a Landry's paralysis developing after antirabies treatment. To decide on the exact nature, a detailed histological examination was undertaken.

Over the cerebrum the meninges showed some hemorrhages, and cellular infiltration with polynuclears and mononuclears. The parenchymal changes were most pronounced in the frontal and ascending parietal area, there was some hyperemia and perivascular infiltration. Polymorphs were found, both perivascularly and diffusely; Babès nodules were present In the thalamus and gray matter underlying the ventricular ependyma there was a perivascular and diffuse infiltration of poly-

lesions gave place to degenerative changes Rabbits and guinea-pigs were injected with the central nervous system of this case, and a virus was isolated The animals became paralyzed on the 3rd day, and died after 5 to 6 days There was some doubt as to the precise nature of the virus, it was probably not a *virus fixe* strain, but resembled more *virus renforcé*.

This case was undoubtedly one of street virus infection with landriform symptoms Had pathological and biological tests not been carried out, it would probably have been regarded as a case of neuroparalytic accident

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violent cellular reaction. Remlinger even suggests that *virus fixe* may be the etiological agent of the milder recoverable, as well as the severer fatal, cases. He would not attribute accidents following the use of killed vaccine to *virus fixe*.

Discussion.

Neuroparalytic accident is a most interesting condition. It must, however, be emphasized that the fact that it may occasionally occur must never be advanced as a contraindication to the administration of antirabies treatment to all exposed to the risk of rabies.

The condition is probably not unique in medicine, for it appears to be related to true postvaccinal encephalitis, and encephalomyelitis complicating smallpox, measles, influenza, and perhaps chickenpox, the fundamental feature of which diseases is the occurrence of perivascular demyelination. Considerable advance has been made in the study of the demyelinating diseases (see Ch. XXXV). It now appears certain that a number of viruses, as well as certain chemical agents and normal nervous tissue, can cause demyelination either by a direct effect on the myelinated tissue, or more probably by first producing a vascular thrombosis The difficulty arises, however, in that demyelination of this characteristic perivascular type has not frequently been found in cases of neuroparalytic accident complicating antirabies treatment. We are reluctant, therefore, to emphasize unduly the analogy between neuroparalytic accident and postvaccinal and other types of post-infective encephalomyelitis.

The views of Remlinger (1937 *a*), who has studied this problem for a lifetime, must always receive the most serious attention. He inclines to the view that there is no single cause of neuroparalytic accidents In some cases, especially where live virus is used, he blames *virus fixe* In other cases, mainly where killed vaccines are used, he believes that the etiological agent is probably the normal nervous tissue.

We would conclude by saying that it is most probable that all cases of neuroparalytic accident can be accounted for by supposing that either *virus fixe* or normal nervous tissue may be the etiological agent. Further, in view of recent findings in related disorders, it is likely that these agents exert their effects by producing intravascular thromboses in the central nervous system.

Differential Diagnosis of Paralysis after Antirabies Treatment

When paralytic symptoms develop in a person who has undergone a course of antirabies treatment, various possibilities must be considered.

1 *Paralytic rabies* The case may be one of paralytic rabies, unprevented by antirabies treatment The diagnostic features of such cases are usual incubation period of rabies, depending on the site of the bite, symptoms of paralysis are [illegible] fatal, histologi- [illegible] t virus from the

2. Rage de laboratoire The case may be one of *rage de laboratoire* with the following diagnostic criteria the incubation period may be longer than in neuroparalytic accidents, the disease usually beginning immediately after a course of injections, or up to 40 days later, the condition is fatal, and from the central nervous system *virus fixe* may be recovered

3 *Neuroparalytic accident.* The paralysis may be a true neuroparalytic accident, the diagnostic criteria being onset 13 to 15 days after the beginning of treatment, symptoms of Landry's paralysis, dorsolumbar myelitis or facial paralysis, no hydrophobia or excitement, a high recovery rate, in fatal cases, absence of lesions characteristic of rabies, including Negri bodies, and absence of street or fixed virus in the brain.

4. *Intercurrent paralysis.* The case may be one of intercurrent paralysis from some undetermined cause, these cases are, of course, notified as neuroparalytic ac-

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CHAPTER LXXX

AUJESZKY'S DISEASE (PSEUDORABIES)[1]

PSEUDORABIES is a virus disease of the nervous system which affects a number of animals, such as cattle, horses, sheep, and pigs, under natural conditions. The infection was first described by Aujeszky in 1902, and occurs in many parts of Europe and the American continent. The disease goes by various names, for in addition to the two already mentioned, it may be called "mad itch." The term "pseudorabies" is misleading as the virus has no connection with that of rabies, and the diseases do not resemble each other in any important respect.

The virus is readily transmissible to rabbits and other experimental animals. Rabbits inoculated by various routes usually develop intense irritation of the skin and rapidly scratch or bite away the fur from the affected site. The disease is highly fatal, both naturally and experimentally. Experimental work has been carried out mainly by French and American authors, and much of this is reviewed in an article by Bosio (1938). Other reviews are those of Galloway (1938) and Remlinger and Bailly (1938).

PSEUDORABIES IN MAN

von Rátz (1913) reported that 2 boys had developed pruritus, after wounding

[illegible] These ob-

[illegible] disease was

[illegible] laboratory

[illegible]

subsided, but she complained of a headache, weakness, an urticarial rash on the feet, an aphthous eruption on the gums, and pain in the knees. Examination of the blood showed a hypercholesterolemia (240 mg. per 100 c c), and an eosinophilia (7 per cent).

Experimental observations. The blood serum of the 2nd case (removed at the height of the itching) was injected into 2 rabbits, 1 intracerebrally and the other subcutaneously. After 18 hours both showed the loss of hair so characteristic of infection with pseudorabies, the animals died from the infection. The loss of hair was most marked in the animal injected subcutaneously, all the hair had been scratched out around the site of inoculation. Blood was removed again, 8 days after the accident, but the virus was not recovered.

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[1] Pseudorabies is primarily an animal disease, accordingly, adopting the rule explained in the preface, we shall confine our description to the features of the disease in man. The properties of the virus are only briefly mentioned.

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The histological features in the patient's brain and the first passage monkey were quite typical (Peers, 1940, 1941). The 4th monkey passage material was inoculated in eastern cotton rats, and produced paralytic symptoms The strain was also passed in mice, and has been very widely used in experimental work (Armstrong, 1939 *b*, 1941). The strain after a few passages in monkeys is pathogenic in these animals (Stefanopoulo and Vieuchange, 1946, Bodian and Cumberland, 1947). Monkeys are said to develop a fulminant infection on inoculation of the Lansing strain together with autolyzed mouse brain (Milzer and Byrd, 1947)

THE SK STRAIN

This strain was isolated from the feces of an abortive case on inoculation of monkeys by Trask, Vignec, and Paul (1938 *a*, *b*). Later, Jungeblut and Sanders (1940 *a*, *b*) succeeded in infecting cotton rats; it was then adapted to mice, and lost virulence for the monkey. After 70 transfers in mice, the strain showed an increased pathogenicity for monkeys and guinea-pigs (Jungeblut and Sanders, 1941; Jungeblut, Sanders, and Feiner, 1942).

A fixed strain of cavian passage virus has been developed by intracerebral passage in guinea-pigs

The SK strain, in its early monkey passages, was related antigenically to other strains isolated in the same epidemic, to the Aycock strain, and some 1934 Californian strains, it differed from certain other monkey-pathogenic strains (Trask, Paul, and Vignee, 1939). It was infective by the oral and intracutaneous routes in [illegible]

[illegible]

the Jungeblut SK strain may not actually be of human origin

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This strain was isolated by van Rooyen in a rhesus monkey from a fatal case in a soldier in the Middle East campaign (Schlesinger, Morgan, and Olitsky, 1943). It infects cotton rats and mice.

OTHER RODENT-PATHOGENIC STRAINS

1. *Jungeblut and Dalldorf* (1943) isolated a virus causing paralysis of albino mice, cotton rats, and hamsters from the brain of a mouse found dead in the home of a fatal case of poliomyelitis. Another strain (MM) was isolated from the brain stem of this case, with a similar range of infectivity, although it was more potent for albino mice.

2 *Jungeblut and Dalldorf* (1946) isolated 2 strains (NY 65 and NY Pool II) from house mice in New York by intracerebral inoculation in albino Swiss mice. The strains were related but not identical, and were distinct from Theiler's and other murine strains

3 *Flexner's MV strain* was adapted to the cotton rat by Toomey, Frohring, and Takacs (1943).

4 *Toomey, Takacs, and Weaver* (1945) isolated in cotton rats a rodent-paralyzing virus from creek water

5 *The McG strain* is a weak strain of rodent-paralyzing virus (see Dalldorf and Whitney, 1943).

6 *Milzer and Byrd* (1947) report that human strains can be adapted to mice, hamsters, and cotton rats by suspension of the material in a 10 per cent. suspension of autolyzed mouse brain They were able to adapt a monkey-passed strain (Leon) to mice and cotton rats They also claim to have isolated several strains from human cord and feces by this technique in mice.

SECTION 9. THE POLIOMYELITIS GROUP

CHAPTER LXXXI

THE POLIOMYELITIS GROUP OF VIRUSES

UNTIL a few years ago it was believed that poliomyelitis virus caused infection of man alone. Experimentally, it was thought to be transmissible only to the monkey or chimpanzee. It is now known that certain human strains can be transmitted to cotton rats, mice, and other rodents. Further, a number of agents has been discovered that is closely related in biological properties to the human virus, and causes natural infections in swine, mice, and other rodents.

As an introduction to the study of human poliomyelitis and a full understanding of its essential biological characteristics, it is essential to be familiar with the modern conception of "the poliomyelitis group of viruses." For example, Gard (1943 *a*)[1] has proposed the use of the terms human, murine or mouse, and porcine or swine, types of poliomyelitis virus. In addition, certain other terms are in general use. Monkey-adapted strains are known as monkey or simian strains. Strains causing paralysis in rodents, irrespective of their source of origin, are known as rodent strains. At the moment, the criteria for classification in this group are not clear, and we include provisionally certain strains that may later be shown not to be true members of the group.

The main members of the poliomyelitis group of viruses are as follows

MONKEY-PATHOGENIC STRAINS OF HUMAN ORIGIN

The bulk of strains isolated from human cases of poliomyelitis is transmissible to the monkey and chimpanzee, and cannot be adapted to rodents After establishment in monkeys, such strains are referred to as monkey-pathogenic or simian strains It is accepted that such strains have derived from the human material, for there is no definite evidence that monkeys are ever spontaneously infected with viruses of the poliomyelitis group The term "ordinary strain" also refers to a monkey-pathogenic strain. The best known of these strains are as follows MA (Flexner and Lewis, 1909 *a*), K (Flexner and Lewis, 1909 *a*, *b*), LP (see Levaditi and Hornus, 1932), Rockefeller mixed or MV, Aycock or Ay (Aycock and Kagan, 1927), New York 1931 strain (see Flexner, 1932), Wfd (Trask and Paul, 1936, 1938, German and Trask, 1938); Sacramento (Howitt, 1937 *a*), McK (Kessel, Stimpert, and Fisk, 1938), Peiping No. 1 (Yen and Hsu, 1941), Mar, isolated in Australia in 1937 and used by Burnet and collaborators

RODENT PATHOGENIC STRAINS OF PRESUMED HUMAN ORIGIN

A number of strains has been isolated with the property of causing infection in rodents The circumstances have been such as to render it more likely that these strains derived from the human material used to initiate the transfers, than from the rodents employed for inoculation.

THE LANSING STRAIN OF ARMSTRONG

This strain was isolated in monkeys by Armstrong (1939 *a*) from the brain and cord of an 18-year-old boy who died of bulbar poliomyelitis in Lansing, Michigan

[1] References are appended at the conclusion of Ch LXXXVIII, p. 1026 et seq

The histological features in the patient's brain and the first passage monkey were quite typical (Peers, 1940, 1941). The 4th monkey passage material was inoculated in eastern cotton rats, and produced paralytic symptoms. The strain was also passed in mice, and has been very widely used in experimental work (Armstrong, 1939 *b*, 1941). The strain after a few passages in monkeys is pathogenic in these animals (Stefanopoulo and Vieuchange, 1946, Bodian and Cumberland, 1947) Monkeys are said to develop a fulminant infection on inoculation of the Lansing strain together with autolyzed mouse brain (Milzer and Byrd, 1947).

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The SK strain, in its early monkey passages, was related antigenically to other strains isolated in the same epidemic, to the Aycock strain, and some 1934 Californian strains, it differed from certain other monkey-pathogenic strains (Trask, Paul, and Vignec, 1939) It was infective by the oral and intracutaneous routes in monkeys at this time (Vignec, Paul, and Trask, 1939). In the Yale laboratory, the original strain, now known as Y-SK has also been found pathogenic for cotton rats and mice, but after this adaptation differs in many properties from Jungeblut and Sanders' SK strain (Melnick and Horstmann, 1947). It is possible, therefore, that the Jungeblut SK strain may not actually be of human origin

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horn cells In addition, there are inflammatory changes consisting of perivascular and focal infiltration with round and glial cells. The nature and distribution of the lesions are very similar to that in mice infected with the Lansing strain

Distribution of virus.

In animals spontaneously paralyzed, virus is present in the brain and cord. In carriers, virus is present in the intestinal tract, to a lesser extent in the intestinal wall, and mesenteric glands, but not in the viscera or CNS (Olitsky, 1939, 1940 *c*). Virus occurs mainly in the upper part of the small intestine; the viral activity of the intestinal contents exceeds that of the intestinal wall by about ten-fold, it is suggested that multiplication may occur in intestinal organisms (Gard, 1945). On recovery from paralysis, the virus may persist in the cord for a year (Theiler, 1937).

Experimental infection in other animals.

Theiler's virus is not pathogenic to *monkeys* by intracerebral injection (Theiler, 1937, Olitsky, 1940 *b*).

Jungeblut (1943) passed the GD VII strain intracerebrally in *cotton rats*. The virus increased in virulence for these animals and mice, and produced nervous involvement in guinea-pigs and monkeys

Strains of virus

The n:
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Titration of infectivity.

Gard (1940 *b*, 1943 *a*) has studied the problem of the titration of infectivity in considerable detail, and has introduced an "incubation time" method for use with the strains FA and GD VII He also studied the 50 per cent. end point titration method, and found that the relation of this titer to the actual virus concentration was dependent not only on the resistance of the cells of the test animal to invasion, but on the physical state of the virus particles The incubation time method was largely independent of the latter condition, and gave more reliable information about the virus content He found that one MID as a rule comprised more than one active virus particle.

The interference phenomenon.

Gard (1944) found that brain suspensions from mice surviving an attack induced by a strain of low virulence, protected against infection when injected intracerebrally together with a more active strain (see p. 916 for more details).

Morphology

Estimates of the particle diameter by filtration are 13–19 mμ (Theiler, 1937), or 9–13 mμ
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Gard
the electr
15 mμ T
tendency to aggregate end-to-end, so that fibers were formed of about 1 μ in length with 8–9 particles From sedimentation and diffusion data he (1943 *a*) estimated the size as being 12–13 mμ

Cultivation.

Gard (1943 *a*) failed to grow the virus in *tissue culture*, but Parker and Hollender
brain suspended in ox

invades the body and
947), the yolk sac and
that virus proliferated

THEILER'S ENCEPHALOMYELITIS OF MICE

The disease was first noted by Theiler (1934, 1937) in the mouse stock at the Rockefeller Institute, New York. He found that certain members of the stock were spontaneously paralyzed, and showed evidence of encephalomyelitis A virus was isolated from the CNS of these animals by intracerebral injection in apparently normal mice.

The infection has many points of resemblance to poliomyelitis in man and is of considerable interest, particularly from the point of view of epidemiology and ecology (see Theiler, 1941; Gard, 1943 *a*).

Clinical features.

The spontaneous disease affects young mice, aged 4-8 weeks, who show flaccid paralysis, commonly of the hind limbs. The incidence of paralytic disease in infected mouse stocks is very low, probably under 1 per 1000, although almost all the members may be intestinal carriers (see below).

Epidemiology.

The disease has been reported not only in North America, but in South America (Roca-Garciá, 1944), Germany (Gildemeister and Ahlfeld, 1938), Japan (Iguchi, 1939), and Sweden, Palestine, and Uganda (see Gard, 1943 *a*).

The infection was originally described in albino mice, but Olitsky and Schlesinger *(1941 b) found intestinal carriers among a number of wild gray mice caught in the* neighborhood of infected albino mice.

Gahagan and Stevenson (1941) isolated from normal mouse stocks a virus closely resembling, but apparently not identical with, that of Theiler.

The virus is widely distributed in members of infected stocks. It is excreted in the feces, and the infection is spread from animal to animal by this means (Olitsky, 1939). *Suckling mice do not show virus in the feces, but it first appears at about 20 days of age;* by 2 months the majority of animals is infected, young mice harbor more virus than older animals (Olitsky, 1940 *a;* Theiler and Gard, 1940 *b*).

The virus has also been detected in the intestines of kangaroo rats *(Dipodomys merriami)* housed near albino mice carrying the virus (Melnick, 1943 *b*)

Experimental infection in mice.

Mice are infected most readily by the intracerebral route, but can also be infected nasally, by injection into the tongue, or intraperitoneally (Theiler, 1934, 1937, Theiler and Gard, 1940 *a;* Olitsky and Schlesinger, 1941 *a;* Young and Cumberland, 1943) Flaccid paralysis develops after an incubation period of 7-30 days.

Jungeblut (1944 *b*) found that rapid passage through mice endowed the strain with an enhanced power of invading the CNS from the periphery.

Young mice are more susceptible than older animals Animals under 4 weeks of age may die without developing paralysis Older animals may suffer from an inapparent *infection On recovery, a residual paralysis of the hind legs may follow*

Mice fed a diet deficient in thiamine showed a lower incidence of infection than controls; in some cases, when deficient animals were later given adequate thiamine they became paralyzed (Rasmussen *et al*, 1944). A less marked decrease in susceptibility was [illegible] in calories Deficiency of riboflavin had no effect [illegible] and Lichstein, 1944) [illegible] y in calcium pantothenate exhibited a definitely [illegible] 1944). Deficiencies in pyridoxine, inositol, and biotin had no consistent effects on the susceptibility of mice (Lichstein *et al*, 1945)

Histology.

The histological features of the natural and experimental disease are similar (Theiler, 1934, 1937, 1941, Olitsky, 1939, Olitsky and Schlesinger, 1941 *a, c*)

The most characteristic lesion is necrosis of the ganglionic cells in the anterior horn Intranuclear inclusions may be seen in the early stages Isolated ganglion cells in the cerebrum and midbrain may also be attacked Neuronophagia is not such a pronounced feature as in the *human being or monkey, and the infection seems to spare more anterior*

TABLE 37[1]

COMPARISON BETWEEN THE VIRUSES OF HUMAN AND MOUSE POLIOMYELITIS AND THE TESCHEN DISEASE (FROM GARD, 1943 *a*)

	Human Poliomyelitis	*Mouse Encephalomyelitis*	*Teschen Disease*
Clinical picture	Preparalytic stage of fever and meningeal symptoms	Not known	Fever and meningeal symptoms
	Paralytic stage: flaccid paralysis, mainly of the limbs	The same	The same
	The ascending type of paralysis common	The same	The same
	Cerebral forms exist, with ataxia and spastic symptoms	Cerebral forms exist, tonic-clonic convulsions main symptoms	Cerebral forms common in young animals, spastic symptoms
	Nonparalytic forms exist	The same	The same
	Incubation period of experimental disease, usually 10–14 days	The same	The same
Pathology	No gross lesions	The same	The same
	Histological lesions confined to the CNS	The same	The same
	Primary process: neuronal disintegration, neuronophagia	The same	The same
	Secondary features: glial reaction, round cell infiltration, perivascular cuffing	The same	The same
	Principal site of lesions anterior horns of the spinal cord	The same	The same
Epidemiology	Annual morbidity rate about 1:10,000 Epidemic attack rate sometimes up to 10 p c	Attack rate about 1 5,000	Epizootic attack rate up to 50 p c
	Age distribution prevalence of children and young adults	Prevalence of young mice	Prevalence of young animals
	In temperate climates characteristic seasonal distribution	Seasonal distribution even	Cold-wet seasons preferred
	Portal of entry of virus not definitely known, probably alimentary tract, principally intestine	Not known, probably intestine	Not known
	Elimination of virus principally feces	The same	Not known
	True contact cases probably rare	The same	The same
Immunology	Reinfections are very rare	Solid immunity after recovery from spontaneous or experimental infection	The same
	Immunologically different strains of virus seem to exist	The same	Not known
	Neutralizing substances appear in the blood with age, apparently no connection with clinical disease	The same	Not known
	Attempts at precipitin reaction and complement fixation unsuccessful	The same	Not made

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Concentration and purification.

Suspensions diluted beyond the infectivity point can be concentrated by pervaporation (Melnick, 1942).

Gard, using a method of purification involving ether, 40 per cent saturation with ammonium sulfate, ultrafiltration, and fractional ultracentrifugation, has isolated a protein material from infected mouse brain which seems to represent the actual virus (Gard and Pedersen, 1941; Gard, 1943 *a*). This work is fully described on p 1004.

He has isolated from the stools of normal white mice a substance he calls "intestinal protein" When isolated from young animals, this substance is infective, but a similar material from older animals is not.

Some other properties of the virus.

In nerve tissue, virus is markedly reduced in infectivity in 30 minutes at 45° C, and milk exerts a protective effect for 5-10° C degrees extra, in intestinal content, virus is completely destroyed at 55° C., irrespective of the suspending fluid (Lawson and Melnick, 1947).

The virus survives for at least 150 days in 50 per cent. glycerol (Theiler, 1937). It is precipitated by ammonium sulfate, and has an optimum pH stability at pH8 or 33 (Theiler and Gard, 1940 *a*). Trask, Melnick, and Wenner (1945) calculated the amounts of chloramine and hypochlorite needed to lower appreciably the infective titer. Brain homogenates show a marked inhibition of glucose phosphorylation (Racker and Krimsky, 1947).

Theiler's virus can be recovered from adult *Musca domestica* 12 days after infection by feeding (Bang and Glaser, 1943).

Immunity.

On recovery from a spontaneous or experimental paralytic or inapparent attack, animals are resistant to inoculation (Theiler, 1937, Olitsky, 1939). Intraperitoneal or intranasal administration of virus gives rise to active immunity, as does the intracerebral injection of an avirulent strain (Theiler and Gard, 1940 *a*) Active immunity is also acquired by healthy carriage of the virus in the gut, probably because some virus is absorbed and reaches the lymph glands

Suckling mice show antibodies in their sera, presumably these antibodies are derived from the mother, as they disappear by 3 weeks, in older animals, virus neutralizing antibodies have again appeared (Olitsky, 1940 *b*, Theiler, 1941). Casals and Palacios (1941) described a complement fixation reaction between antisera and infected brain tissue Human sera only rarely neutralize virus (Seligmann and Jungeblut, 1943, Olitsky and Findlay, 1946)

Relationship to other members of the poliomyelitis group

The antigenic relationship of Theiler's virus to other members of the poliomyelitis group is fully discussed below

Gard (1943 *a*) concludes that mouse encephalomyelitis and human poliomyelitis are closely related In his opinion there are no essentially distinguishing features between the two, except the species specificity The two viruses should be regarded as variants or types of the same species, until evidence to the contrary is produced Table 37 from Gard's (1943 *a*) paper presents the main features of the 2 diseases along with those of the Teschen disease of swine (see below)

THE TESCHEN DISEASE

Gard (1943 *a*) gives the following description The Teschen disease of pigs or *Encephalomyelitis enzootica suum* was first recognized in Czechoslovakia in 1929, but is now more widespread in Europe Certain forms of the disease are characterized by flaccid paralysis Histologically, there is loss of ganglion cells, and mesodermal-glial reaction in the anterior horn cells and base of the brain The disease can be transmitted to young swine by intracerebral injection There is no doubt that the disease has certain points of resemblance to human poliomyelitis and Theiler's disease, although there are differences (see Frauchiger and Hofmann, 1941).

(Jungeblut, 1944 *a*; Dalldorf and Whitney, 1945, Melnick and Horstmann, 1947). In all probability, certain murine strains share antigenic components with simian strains (Jungeblut, 1944 *a*, Milze and Byrd, 1947). Thus, Toomey and Takacs (1941 *c*) found the Lansing strain to be neutralized by convalescent antiserum in the same manner as were monkey strains (see also Harford and Bronfenbrenner, 1941). Experiments with hyperimmune monkey serum indicated a relationship between the McK and MV monkey strains and the Lansing strain (Kessel, Moore, and Pait, 1946). Jungeblut, Sanders, and Feiner (1942) showed that SK was neutralized by antiserum prepared in the horse to a monkey strain, however, antiserum to the murine strain did not inactivate the monkey strain. They concluded that SK and other murine strains should be regarded as variants of the monkey-pathogenic type of virus.

As regards Theiler's virus, it appears to be antigenically distinct from monkey strains, as shown by Theiler (1937). Olitsky (1940 *b*) found that Theiler's virus was not inactivated by antiserum from convalescent human beings or monkeys; *vice versa*, serum from mice convalescent from infection with Theiler's virus did not neutralize monkey virus. There does not seem to be any relationship between Theiler's virus and the Lansing strain (Young and Cumberland, 1943; Levaditi and Vaisman, 1945).

Dalldorf and Whitney (1945) using cross neutralization and cross immunity tests, found no relationship between MM virus and Theiler's original TO strain, complement fixation, however, showed a relationship between the strain GD VII of Theiler and the MM virus

Jungeblut, Sanders, and Feiner (1942) found that Theiler's virus was neutralized by antiserum to the murine SK strain, and to a lesser extent by antiserum to a monkey strain. However, mice recovered from infection with Theiler's virus were fully susceptible to infection with SK Later, Jungeblut (1943) in cross neutralization tests, could not demonstrate any relationship between Theiler's virus and the SK strain Melnick (1943 *b*) came to the same conclusion using complement fixation tests The Y-SK strain is not related to Theiler's virus (Melnick and Horstmann, 1947) Mice immunized against the monkey strain Leon adapted to mice were not resistant to inoculation with the FA strain of Theiler's virus (Milzer and Byrd, 1947)

We may conclude that all the viruses referred to show some antigenic relationship, and 3 broad groups can be defined (1) monkey-pathogenic strains; (2) Lansing, SK, MM, and probably certain other strains, (3) Theiler's virus In group 2, the relationships one to another are probably closer than to groups 1 or 3. The relationship between groups 1 and 2 is probably closer than between 1 and 3 or 2 and 3

ANTIGENIC RELATIONSHIP OF THE POLIOMYELITIS GROUP TO OTHER VIRUSES

Vaccinia Infection with neurovaccinia did not modify the characteristic lesions of poliomyelitis, and vaccinia did not "activate" a weakly active strain of poliomyelitis (Hurst and Fairbrother, 1931) Poliomyelitis is not neutralized by vaccinial antiserum (Jungeblut, 1934)

Foot-and-mouth disease No cross immunity was found between the neurotropic strain and Theiler's virus (Levaditi and Vaisman, 1945).

antirabic sera (Jungeblut, 1934).

neutralized by antiherpetic sera

Lymphocytic choriomeningitis. There is no evidence of a serological relationship between the SK strain and the virus of LCM (Jungeblut, Sanders, and Feiner, 1942, Sanders and Jungeblut, 1942).

COMPARISON BETWEEN THE VIRUSES OF HUMAN AND MOUSE POLIOMYELITIS AND THE TESCHEN DISEASE (FROM GARD, 1943 *a*)—*Continued*

	Human Poliomyelitis	*Mouse Encephalomyelitis*	*Teschen Disease*
Distribution of virus	In clinically manifest cases: virus in the CNS but no other tissues except the intestinal wall and sometimes mesenteric lymph nodes	The same	Virus in the CNS, intestines with contents not examined
	Healthy carriers harbor virus in the intestines, to what extent not known	Carrier state is the rule	Not known
Properties of the virus	Infectious spectrum narrow: man, certain primates, occasionally cotton rat and mouse	Infectious spectrum narrow: albinotic mouse, gray mouse, occasionally cotton rat	Ordinary laboratory animals refractory
	Under experimental conditions strictly neurotropic	The same	The same
	Generally not cultivable in tissue culture	The same	No attempts
	Filtration end-point 30 mμ	Filtration end-point: 30 mμ	Not studied
	Remarkable resistance in vitro	The same	Not studied

ANTIGENIC RELATIONSHIPS IN THE POLIOMYELITIS GROUP

Antigenic Structure of Monkey-Pathogenic Strains

Flexner (1932) drew attention to antigenic differences between human (i.e., freshly isolated) and monkey-passed strains, passage through monkeys modified antigenic structure so that antisera detected quantitative and qualitative differences, monkey virus still induced active immunity to human virus Paul and Trask (1933), however, found that animals recovered from infection by a human strain were not protected against passage virus, and vice versa. Human convalescent sera showed a qualitative difference in neutralizing power against human and passage strains

Other workers, using cross neutralization tests, have noted degrees of antigenic differences between human and passage strains (e.g., Burnet and Macnamara, 1929, 1931; Weyer, 1931-2, Southby and McKie, 1933, Erber and Pettit, 1934, Kessel *et al*, 1936-7, Howitt, 1937 *a;* Burnet and Keogh, 1938, Kessel, Stimpert, and Fisk, 1938). Thus, Burnet and Macnamara found that monkeys recovered from infection with their Australian strain succumbed to the MV strain Howitt reported that antiserum prepared in monkeys to the Sacramento strain did not protect against passage material, while certain animals immune to Sacramento virus succumbed to passage virus. It is evident, therefore, that antigenic differences can be detected between human and monkey-passed strains

Freshly isolated strains can also vary one from another Thus, Trask *et al* (1937 *a*, *b*) found that immunological differences existed between human strains isolated in various outbreaks in 1931 and 1934 (see also Stimpert and Kessel, 1939) Trask, Paul, and Vignec (1939) also detected differences between human strains Kessel, Moore, and Pait (1946) found antigenic differences between strains, unrelated to the number of passages.

ANTIGENIC STRUCTURE OF MURINE STRAINS AND RELATIONSHIP TO SIMIAN STRAINS

Neutralization and cross immunity tests have shown that the SK, Y-SK, MM, and Lansing strains are related, and probably share common antigenic components

He found that if mice survived infection with a strain of low virulence, they became immune, and their brain suspension would protect other mice against infection, if injected intracerebrally along with active virus. A less pronounced protective effect was demonstrated when brain was given intraperitoneally. The protective power was not dependent on the virus content, and was attributed to the presence of a specific inhibitor. This inhibitor was attached to the tissue component of the suspension, and was not soluble in water or saline. It did not combine with virus *in vitro* It appeared with the onset of symptoms.

Canine distemper. Rhesus monkeys infected with distemper virus are immune to poliomyelitis, but this resistance disappears in convalescence (Dalldorf *et al.*, 1938).

Epidemic keratoconjunctivitis. No relationship was found between Theiler's virus and the agent of this disease (Sanders and Alexander, 1943).

Roca-García's viruses. The 3 viruses isolated by Roca-García (1944) did not immunize mice definitely against Theiler's virus.

OTHER BIOLOGICAL RELATIONSHIPS OF THE POLIOMYELITIS GROUP

Simultaneous Infection with Poliomyelitis and Other Viruses

Lymphogranuloma. When mice infected with LGI were injected with poliomyelitis virus, the lesions characteristic of LGI developed as usual, when the two viruses were mixed *in vitro* prior to inoculation in mice, the lesions of LGI developed, but when inoculated in monkeys those of poliomyelitis were noted (Vieuchange, 1936 *c*).

Rabies. Rabies virus and the Lansing strain can be passed together intracerebrally for several passages, because rabies attacks the brain and poliomyelitis the cord (Levaditi, 1943).

The Interference Phenomenon

The interference phenomenon was first demonstrated in poliomyelitis by Dalldorf (1939), who noted that the virus of lymphocytic choriomeningitis had a "sparing" effect on experimental poliomyelitis in the monkey; this effect was reciprocal to a lesser degree Later it was found that hamsters injected intracerebrally with LCM virus failed to develop symptoms on intraperitoneal injection of the MM strain (Dalldorf and Whitney, 1943), a similar interfering effect was produced by the rodent paralyzing strains McG, Lansing, and SK.

Jungeblut and Sanders (1940 *a*) showed that interference occurred on simultaneous intracerebral inoculation in the monkey of the murine SK strain and the original monkey SK strain; interference also occurred if either was given 48 hours before the other.

The murine SK strain, in the form of a suspension of mouse brain, was found to interfere with the propagation of the Aycock and RMV monkey strains in rhesus monkeys (Jungeblut and Sanders, 1942). The mixture could be prepared *in vitro*, or the two agents could be injected separately, by different routes. Interference did not occur with heated infected brain, normal brain, or herpes-infected brain. If murine virus was inoculated intravenously in monkeys, before or after an intracerebral injection, a distinct interfering effect was observed It was suggested that a blockade of susceptible cells by the nonparalyzing murine virus rendered these cells insusceptible to invasion by the monkey-paralyzing virus.

Tissue-cultured SK virus also showed the interfering effect in monkeys (Sanders and Jungeblut, 1942).

The cavian passage strain of SK, when injected intravenously, did not act as an interfering agent against an intracerebral injection of the monkey RMV strain (Jungeblut, Feiner, and Sanders, 1942)

Jungeblut (1945) further investigated the interference phenomenon in rhesus monkeys, and found that phenolization and formolization rendered the SK strain "noninterfering." Preparations subjected to UVL retained some interfering properties. He was unable to separate the interfering property from infectivity. He also found that the MM strain interfered with the development of monkey strains. Theiler's virus, on the other hand, did not prevent infection due to monkey strains

The interfering agent in the SK or MM strains may be the virus particle itself, as has been shown in the case of influenza On the other hand, the work of Gard (1944) with Theiler's virus suggests that this may not be the correct explanation

ber of excellent articles, reviews, and smaller books of a general nature has also appeared (e g., Flexner, 1912, 1915, Peabody, Draper, and Dochez, 1912; Kling and Levaditi, 1913 *a*, *b*, Draper, 1917, 1935, Amoss, 1928, 1930, Schultz, 1932; Faber, 1933, 1941; *League of Nations Epidemiological Report*, 1935, Brodie, 1938, Perdrau, 1938, Camps, 1940, Toomey, 1940 *b*, 1941 *a*, *b*, 1943 *b*, Bashford, 1941; Paul, 1941; Trask and Paul, 1941 *a*, Bedson, 1943; Brooks, 1943, Fraser, 1943, Gautier and Bamatter, 1943, Wyllie, 1943, Selby, 1945, Sabin, 1947, Nelson, 1947). Fishbein, Hektoen, and Salmonsen (1946) have compiled a very extensive bibliography covering the years 1789–1944, several thousand papers are mentioned, short summaries being given in many cases.

FACTORS PREDISPOSING TO POLIOMYELITIS

As will become evident, it is now known that the virus of poliomyelitis is widely distributed in epidemic periods, especially in the intestinal tract of close contacts. As only a minority of such infected persons appears to develop symptoms, it is important to inquire whether there is any evidence that certain factors may predispose to the development of clinical poliomyelitis.

Physique. Draper (1917, 1932, 1935) has maintained that children suffering from poliomyelitis regularly display certain morphological characteristics. The children are usually large and plump. The face is broad, and in about half the cases the upper central incisors are definitely separated, frequently other teeth stand free also. In older persons, on the contrary, the teeth are often crowded, and maxillary prognathism occurs The subcostal angle may be narrow, and the hand short and broad The pelvis may be wide in relation to the shoulder girdle, males may show poor development of the genitalia.

A very characteristic feature is the shape of the palpebral fissure, the inner half of the upper lid curving downward, and the inner canthus being lower than the outer. The eye is definitely "slit" in appearance and gives a mongoloid look to the face. Pigmented moles or freckles are commonly found on the face, neck, and upper part of the chest. The skin is smooth and hairless.

The whole condition of the patient suggests some widespread endocrine disturbance. Distinctive properties have been found in the leukocytes, grown in tissue culture (Draper, Ramsey, and Dupertuis, 1944). In view of Draper's wide experi-

may play a part in predisposing to attack (Addair and Snyder, 1942, Aycock, 1942 *a*) Armstrong and Davis (1945) found no evidence that paralytic attacks were more frequent in the children of parents who did not readily produce virus

ttee, 1932)

ce of paralysis may be due to

is inclined to occur in (

predispose to the dise

vitamin C predisposes to infection and severity of attack. Hypoglycemia may be a factor in determining susceptibility (Sandler, 1941).

Trauma There is a fairly general impression that overexertion or other more specific injury predisposes to infection (see, e g, Lovett and Lucas, 1908, Batten, 1910, Wayson, 1918 *a*, Leake, 1936 *a*, Muller, 1941).

Parker (1937) reported 3 cases where following a fall, or a blow to a limb, poliomyelitis developed with the main incidence in the traumatized limb Martin

CHAPTER LXXXII

ACUTE ANTERIOR POLIOMYELITIS (INFANTILE PARALYSIS)

Historical

PALEOPATHOLOGY shows that poliomyelitis existed in ancient Egypt. During the course of archaeological excavations south of Cairo, Flinders Petrie (International Committee, 1932) unearthed a skeleton (dating from about 3700 B C.), the left leg of which was much shorter than the right, probably caused by the disease. Other

nasty

iphis

, the

foot of which is shown lying in the typical equinus position.

A number of cases of paralysis associated with muscular wasting was recorded in the 18th and 19th centuries both in the British Isles and elsewhere, of which those recorded in England by Badham (1836)[1] should be specially mentioned. In a paper entitled "Four Remarkable Cases of Suddenly Induced Paralysis in the Extremities" he described illnesses in young children which were very suggestive of poliomyelitis as we now know it. In certain cases there were some preparalytic disturbances, but in others paralysis was the first manifestation. Badham was of the opinion that this was a disease *sui generis* and unrelated to other conditions known in his day. His paper was instrumental in stimulating Heine to publish a book on the subj[illegible] of the

disease.

quently

ment of

limbs.

Cases were recorded by a number of other workers after interest had been aroused by Heine's writings. Medin (1891, see also 1896) recorded the features of a large Swedish outbreak, and first convincingly showed that the attack of the pathogenic agent was not limited to the cord only, but that the higher centers might be involved In recognition of their pioneer work the disease is often referred to as the Heine-Medin disease.

Epidemics of the disease became extremely prevalent from the end of the 19th century onward, and many theories were current regarding the etiology, that it was an infective condition was suggested by Wickman's work on the epidemiology (1907). This view was proved to be correct by Landsteiner and Popper (1908, 1909) who succeeded in infecting a monkey with human material. It soon became evident, largely by the researches of Flexner and his colleagues in America, that the pathogenic agent was a filtrable, uncultivable virus.

Since the beginning of this century our knowledge of the virus agent has increased greatly, but there are still aspects of the epidemiology and immunology of poliomyelitis that await elucidation

The disease has always attracted the greatest interest in the literature, and we have estimated that well over 10,000 papers have been published In the face of such a mass of literature, it is obviously impossible to mention any save the most important contributions to our knowledge. The standard reference work on poliomyelitis is the *Report of the International Committee* (1932), and those desiring fuller information on any point should consult this most complete volume A num-

[1] References are appended at the conclusion of Ch LXXXVIII, p 1026 et seq

liminary stage of general infection. Many now hold that the earlier manifestations of infection are due to the presence of ... 'she, 1933). Others have postulated a prelimi... by subarachnoid and meningeal invasion (...

CLASSIFICATIONS

Wickman (1907, 1913) recognized the following varieties spinal, landriform,

lower motor neuron paralyses

The classification of the International Committee (1932) is as follows (1) Abortive type with no constitutional symptoms and no evidence of CNS involvement. (2) Nonparalytic type with transient weakness probably due to edema of ...cortical paralysis, ...) Cerebral or en-... (5) Ataxic type, probably due to involvement of the cerebellum, Clarke's column, and the intervertebral ganglia

Another classification is that of Faber (1933), and suggested earlier by Peabody, Draper, and Dochez (1912) and Draper (1917). The disease is divided into 4 phases the first 2 occur early and late in the preparalytic stage, the 3rd phase is that of paralysis, while the 4th phase is that of recovery

Powell (1938) recognized ... febrile phase without paraly... paralysis in the second, (5) ... (6) an acute febrile illness without paralysis.

Adamson and Dubo (1942) describe 3 types characterized by. (1) no paralysis or changes in the CSF, but typical clinically; (2) no paralysis, pleocytosis present, clinically typical, (3) paralysis present

The International Committee differentiates between 2 types of preparalytic poliomyelitis, the abortive type occurring early, and the nonparalytic type occurring later More recently it seems that the terms "abortive" and "nonparalytic" have become synonymous

ILLNESS OF INFECTION

There may be a transient minor illness within a day or so of the presumptive date of infection, characterized by nasopharyngitis, fever, and headache (International Committee, 1932, Abegg, 1942, Fanconi and Zellweger, 1942, Conybeare, 1946, McFarlan, 1946, McFarlan, Dick, and Seddon, 1946).

It is probable that persons may show these symptoms at the time of exposure to infection, without necessar...

After 1 to 2 days of syr... patient recovers and appears ... may supervene It has been ... illness (see below) can be explained on the basis of the first febrile "hump" being an illness of infection, and the second "hump" denoting the end of the incubation period, and the beginning of invasion (Paul, see International Committee, 1932, McFarlan).

The illness of infection is usually caused by the poliomyelitis virus itself, but in some cases as mentioned above may be due to other infectious agents

NONPARALYTIC (ABORTIVE) CASES

An increasing interest has been taken in these cases of recent years, for in any epidemic there are likely to be several times as many nonparalytic as paralytic

(1933) has described cases of poliomyelitis following on a simple fracture (see also Zenke, 1939).

Exposure to extremes of temperature, wetting, and operations may act as predisposing factors (see Muller, 1941; Keller, 1942). Salt-depletion and hemoconcentration have been blamed by Rinehart (1944). Faradic stimulation in the incubation period was not found to influence the site of initial paralysis but it caused a severer lesion (Swan, 1941).

Infections. Some authors suggest that poliomyelitis often supervenes on another infection such as measles, chickenpox, whooping cough, bronchitis, enteritis, or sore throat (International Committee, 1932; Muller, 1941; Zeller, 1942; Zellweger, 1945) There may be some relationship between susceptibility to diphtheria and poliomyelitis (Heaslip, 1938 *b*).

Tonsillectomy. There has been a large number of cases reported where bulbar or bulbospinal poliomyelitis has developed within 7 to 30 days of tonsillectomy or adenoidectomy (Aycock and Luther, 1929, Ayer, 1929, Brown, 1931–2, Fischer and Stillerman, 1937, Anderson and Dixon, 1938, Eley and Flake, 1938, Kramer, Gilliam, and Molner, 1939, Fischer, Stillerman and Marks, 1941, Helms, 1941 *a*, Aycock, 1942 *b*, Toomey and Krill, 1942, Zellweger, 1943, Howard, 1944, Page, 1944,

Bahlke, 1947).

In these cases either virus is present in the throat at the time of the operation, or else it is deposited there later, while the tissues are still traumatized Virus presumably ascends to the bulb via nerves exposed at the operation. A tragic instance of this condition has been recorded where tonsillectomy was performed on 5 apparently healthy children, who all contracted bulbar poliomyelitis (Krill and Toomey, 1941, Francis *et al.*, 1942. It is probable that tonsillectomy or adenoidectomy may predispose the patient to infection even weeks later (Aycock, 1942 *b*, Lucchesi and LaBoccetta, 1944)

It is possible that peripheral trauma may cause changes in the brain or cord around the central connections of the nerves supplying the affected area, if virus is already present in the CNS, it may be activated by such changes (Horstmann and Paul, 1947)

The onset of these cases is sudden, with sore throat, cough, and pyrexial disturbance There are paralytic phenomena such as strabismus and ptosis, facial paralysis, and dysphagia, trismus is also present The main histological changes occur in the bulb.

Pregnancy There is little doubt that pregnancy predisposes to poliomyelitis, and that there is a higher incidence in pregnant women than in a control group otherwise comparable (McGoogan, 1932, Aycock, 1941, 1946, Helms, 1941 *a*, Harmon and Hoyne, 1943, Blair and Robertson, 1944, Dhunér, 1944, Fox and Sennett, 1945, Horstmann, Ipsen, and Lassen, 1946, Fox and Waisman, 1947). The fetus is not affected by the disease.

INCUBATION PERIOD

The incubation period is 7 to 10 days In the 1941 epidemic in Alabama, Casey (1942 *a*) studied 37 cases developing following the visit of a prior victim, the incubations varied from 5–35 days, with an average of $12 \cdot 2 \pm 1 \cdot 1$ days

CLINICAL FEATURES AND COURSE OF THE DISEASE

The conception of poliomyelitis has changed greatly in recent years For one thing, nonparalytic cases are now recognized to be very much commoner than frankly paralytic ones For another, Hurst's work on the pathogenesis (see below) of experimental poliomyelitis has caused many to abandon the older view of a pre-

The above description is that of the most commonly observed features of preparalytic cases, without particular reference to time. According to Faber (1933) the clinical features can be described in two phases from the point of view of time.

Features of Phase 1. Here, the symptoms are thought to be due to involvement of the brain stem and basal nuclei. The earliest symptoms are those either of drowsiness and listlessness or of restlessness and irritability. Fever is usually present. There are frequently psychic disturbances in which the sleep rhythm may be inverted, and the child hyperesthetic. Gastro-intestinal disturbances, vomiting, anorexia, and constipation are common. A number of signs such as flushing, quick pulse, and irregular action of the heart may be noted. There is no loss or clouding of consciousness, the cerebrum being relatively unaffected by the virus. It is suggested that these various disturbances of the first phase are most readily explicable by their having a subcortical origin in the thalamus (e g., hyperesthesia) or hypothalamus (e g, sleep, fever, disturbances of the vasomotor system). Even at this early stage, there may be changes in the cerebrospinal fluid (see below). Some cases may not develop any further, but many progress to Phase 2.

Features of Phase 2. The thalamic and hypothalamic disturbances of Phase 1 persist, but as the virus spreads nearer to the cord, sensory symptoms develop, due to involvement of the posterior columns This phase is sometimes known as "posterior poliomyelitis." The neck is now stiff and rigid, and there is muscular ataxia and tremor, while the reflexes may be increased. Pains may be spontaneous, or produced by touching and handling the child It is suggested that this phase is due to an involvement of the posterior columns and root ganglia, and not to meningeal irritation

The majority of preparalytic cases runs through both these phases of illness, but does not progress any further. Phase 1 may merge gradually into Phase 2, or they may be separated by a distinct interval, and when this is so they are known as "dromedary" cases, from the two humps of reaction separated by a more or less normal period. The average length of the preparalytic period is about 4 days.

Dromedary cases were originally described by Draper in 1917 and have been noted by other authors (e.g, Macnamara, 1928; Macnamara and Morgan, 1932; Gard, 1937; Hunt, 1938, McAlpine, 1945, 1946). They appear to be commoner in some outbreaks than others. The duration is variable, being often as much as 14 days Thus for 2 to 3 days the patient is drowsy, febrile, may vomit, and may have an upper respiratory catarrh. Thereafter improvement sets in for up to 7 days, to be succeeded by 1 to 2 days of definitely nervous involvement, with neck rigidity, the "spine sign," pains, tenderness, and other features as described above.

The dromedary, or biphasic type of illness has been said to be of bad prognostic [illegible]

common at the beginning than the end of an epidemic (5 per cent. as against 60 per cent of all cases), and suggested that the shorter the time between the two phases the severer the attack.

There are at least 2 interpretations for the dromedary case (see Horstmann and Paul, 1947). Either the incubation period is the interval between exposure and the first hump, or the interval between the two humps, the first being in the nature of an illness of infection.

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sec [illegible]

other infections, and appropriate laboratory tests may have to be employed to make the distinction Lymphocytic choriomeningitis and "serous meningitis" (Brodie,

cases. In an outbreak in New Haven in 1931, Paul, Salinger, and Trask (*1933 b*) found that 5 to 8 per cent. of children under 10 years old (of a selected group) had frank paralytic poliomyelitis, while 30 to 40 per cent. had nonparalytic poliomyelitis. Abortive cases are commonest below 16 years of age (Paul, Salinger, and Trask, *1933 b*).

The diagnosis in the preparalytic stage presents a difficult problem especially in nonepidemic periods. The clinical features have been described frequently since the days of Wickman (1907), (e.g., Muller, 1910, Peabody, Draper, and Dochez, 1912; Frost, 1913, Draper, 1917, Zingher, 1917 *b*; Macnamara, 1928, Kramer and Aycock, 1931–2; Gordon, 1932, Paul, Salinger, and Trask, 1932, *1933 a, b*, Macnamara and Morgan, 1932; Trask and Paul, 1933; Faber, 1933; Martin, 1933, Walshe, 1933, Baastrup, 1935; Siegl, 1936, Bingold, 1936; MacNalty, 1936, Muller, 1936; Nissen, 1936, Gard, 1937; Lucchesi, 1937, Stender, *1937*, Wieland, *1937*, Aguirre, 1938; Gsell, 1938, Holst, 1938, Stahel, 1938, Hamilton, 1940, Adamson and Dubo, 1942, Agius *et al.*, 1945, Debono, 1945; McAlpine, 1945, 1946).

Preparalytic poliomyelitis begins in various ways. Fever is usually present for 1 to 4 days. "Influenzal" and catarrhal symptoms are common, with sore throat, tonsillitis, and nasopharyngitis. Frontal headache is frequent. In another group, the onset is with gastro-intestinal symptoms of diarrhea, vomiting, and abdominal pain. Convulsions may occur. In the Malta (1942–43) outbreak there was no obvious relation between the mode of onset, and the distribution of the subsequent paralysis (Agius *et al*, 1945).

Pain in the head, neck, back, and limbs is common, and is due to a generalized hyperesthesia; it is elicited when any attempt involving movement, especially of the spine, is made (see Debono, 1945). The location of the pain does not usually correspond to the site of subsequent paralysis, and the severity has no relation to the *extent of paralysis (Agius et al., 1945)*.

Stiffness in the neck and back and resistance to anterior flexion are very common. If the child is lying down and the head is gently raised, pain is usually complained of, and rigidity is experienced by the observer. *If the shoulders are* raised, the head usually falls back to the bed. Another test is to sit the child on one's knee and then tempt him with toys. On reaching out he will try to bend his neck forward, and experience pain (Macnamara, 1928). Older children, when asked to bend forward to kiss their knees, will be unable to do so. Another well-known sign is Amoss's or the tripod sign. The child is asked to sit straight up in bed, when he will be found to place his hands on the bed behind himself, for support. The child's arms and trunk thus resemble a tripod in shape (see Hannah, 1937) Kernig's sign is often weakly positive. Muscular tremors often occur, and usually herald the site of forthcoming paralysis The skin may be hyperesthetic, and show vasomotor disturbances, such as flushings and sweatings. Gard (1937) has stressed the importance of a cyanotic tinge of the face with circumoral pallor. The abdominal reflexes are often lost, and the tendon jerks unequal.

The child is usually irritable, restless, and often snarling. Apathy and stupor *may occur in some cases* Gard (*1937*) *found a number of cases* with encephalitic symptoms of somnolence, delirium, ataxia, nystagmus, clonus, and spasms Although many of these progressed to develop definite flaccid paralyses, others did not, and had to be *considered as cases of nonparalytic poliomyelitis* (see also Agius *et al.*) Adamson and Dubo (1942) stress the diagnostic value of the triad of symptoms of spontaneous pain in the head and back, stiffness of the neck and back, and fever

Nonparalytic cases *should be kept in hospital for at least 4 weeks*, as paralysis may develop later (Gard).

Examination of the cerebrospinal fluid is of the very first importance Cases can scarcely be accepted as preparalytic poliomyelitis *unless the cell count is at least* 12 per c mm. The count frequently reaches higher figures, even occasionally 1,000 cells per c.mm (see below).

upper part of the digestive system. He finds that these bulbar cases fall into 5 *groups of increasing grades of severity*.

1. The clinical manifestations are those of a facial paralysis It is suggested that the virus spreads by the chorda tympani to the nucleus salivatorius (adajacent to the 7th nucleus).
2. Both 6th and 7th nerves are paralyzed owing to the proximity of their nuclei It is suggested that the virus spreads from the facial nucleus to involve the abducens nucleus.
3. Other nuclei neighboring the 7th are involved, with more widespread symptoms of bulbar paralysis
4. [illegible] ...ract as well as the facial nucleus, and
5. [illegible] intestines simultaneously, giving rise ... 7th nucleus.

The Minnesota Poliomyelitis Research Commission (1947) studied nearly 200 cases in 1946, and suggested the following classification: (1) bulbar-cranial nerve nuclei group, (2) bulbar-respiratory center group, (3) bulbar-circulatory center group, (4) bulbar-encephalitic group, (5) combined bulbar-cervical cord group

The landriform type of poliomyelitis *may be confused with other forms of* Landry's paralysis The disease may begin in the hip muscles and spread rapidly in both directions, or it may begin in the arms and spread to the neck and down to the legs

The cerebellar type is associated with pronounced ataxia, nystagmus, and incoordination *(see Neal, Du Bois, and Abrahamson, 1916–19, Glanzmann, 1937)* *Meningitic* and *polyneuritic* types are also described

The sympathetic nervous system in poliomyelitis The paralyzed limbs may show vasomotor disturbances, being cyanotic and cold Toomey (1931–2 *c*) found that the superficial abdominal reflexes were lost or diminished before paralysis appeared, and *suggested that this might be due to sympathetic paralysis* He injected paralyzed patients with atropine, histamine, and adrenalin without definite results When pilocarpine was injected, sweating began sooner, and lasted longer, on the skin of the paralyzed limb than the normal (see also Toomey, 1934 *b*) When epinephrine was injected into these patients, sweating ceased at once over the normal limb, *whereas it was unaffected over the paralyzed limb* Twenty-one days after the onset the paralyzed side reacted normally. These results are certainly suggestive [illegible]

[illegible]

later (Phase 2), the abdominal [illegible] changes in the knee jerks, and lastly (Phase 3), the abdominal reflexes and knee jerks are both diminished or lost.

The sense of smell Hyland *et al.* (1938) did not find any abnormality.

Differential diagnosis Numerous cases diagnosed as the Guillain-Barré syndrome of acute infectious polyneuritis have probably been suffering from poliomyelitis (see De Sanctis and Green, 1942, Jones, Holmes, and Weinstein, 1943, Jervis and Strasburger, 1943, Blumberg, Mahoney, and Wenger, 1944, Brazier *et al*, 1944, Nicholson, 1946) Certain neurological infections of undetermined etiology may *mimic poliomyelitis (see Babonneix, 1939–40)*.

THE STAGE OF RECOVERY

A remarkable feature of the disease is that persons profoundly paralyzed, even in all limbs, may recover completely in a matter of a few weeks. Recovery begins

1937; Lassen, 1939); simple infections of the upper respiratory tract (Levin *et al*, 1942); sandfly, dengue, and dengue-like fevers (Bernstein, Clark, and Tunbridge, 1945; Paul, Antes, and Sahs, 1945). Many cases diagnosed as "meningismus" may in reality be abortive poliomyelitis (Illingworth, 1945). In N. America there is some difficulty in making a clinical diagnosis between equine encephalitis, St. Louis encephalitis, mumps meningitis, and abortive poliomyelitis

PARALYTIC CASES

The commonest types of paralytic poliomyelitis are the ordinary or spinal, the polioencephalitic, and the bulbar or bulbospinal types, but certain others are described.

Spinal type. If carefully questioned, the great majority of cases will be found to have shown some evidences of infection in the preparalytic stage. In other cases, [illegible] ikes up with a limb paralyzed. [illegible] after a lapse of about 7 days, [illegible] volved are those of the lower extremity, especially the anterior tibial and other groups below the knee. In severer cases, the muscles of the pelvic girdle may be affected, making it impossible for the child to flex the hip joint. The abdominal and intercostal muscles may be involved, as may the diaphragm, and in such cases the outlook is very serious, death usually occurring from respiratory paralysis When the arms are paralyzed, this is usually more marked in the proximal than in the distal groups. Nelson (1946) found that there was a significant predominance of paralysis in the left, as compared with the right, arm. Paralysis of the neck muscles is often associated with respiratory paralysis, and is of very bad prognostic import. The characteristic changes in the reflexes and in the cerebrospinal fluid are described separately. Localized pain may occur, sometimes associated with muscular spasm Pain and tenderness may be found in nonparalyzed as well as paralyzed muscles, because the inflammation of the sensory neurons does not necessarily occur at the same level as the changes involving the anterior horn cells (see Moldaver, 1944). By means of the pathesthesiometer it has been shown that sensory changes occur, in the nature of a lowered threshold of vibratory sensibility (Frohring *et al*, 1945) Nelson (1946) draws attention to disturbed circulation in the involved extremities

Polioencephalitic type Despite suggestions to the contrary (see Martin, 1933) there is no doubt that the virus can cause a true encephalitis The neurological signs differ from those commonly observed in spinal poliomyelitis, in that convulsions and other cerebral manifestations occur

The bulbar type of poliomyelitis is associated with cranial nerve paralysis The facial nerve is most commonly affected, the abducens and other ocular nerves are more rarely involved This type of illness usually carries a high mortality rate, and may be associated with paralysis of the limbs and trunk. Cranial nerve paralysis has been noted more commonly in some outbreaks than others (Ruhrah, 1917, Bull, 1937–38, Sherman, 1944, Nicholson, 1946).

In the Malta 1942–43 outbreak, 11 per cent of native cases showed only a facial paralysis (Agius *et al*, 1945), while 28 per cent of service cases showed cranial nerve palsy (Bernstein, Clark, and Tunbridge, 1945) Wyllie (1944) has described a small family outbreak, 1 person developing paralytic poliomyelitis, and 2 persons Bell's palsy Stebbins, Gillick, and Ingraham (1939) described an outbreak of 20 cases, 13 of whom showed bulbar symptoms, only 2 had had a tonsillectomy, 12 died In the 1946 Minneapolis epidemic, Pohl (1947) found that a complaint of sore throat was 10 times more frequent in cases in which bulbar features eventually developed than in those which later showed paralysis of spinal origin

Some bulbar cases may develop after tonsillectomy (see above) Toomey (1936 *a*) has made a study of bulbar cases, and has suggested that virus reaches the bulb by the chorda tympani fibers in the facial nerve, that is to say, from the

MORTALITY

Death is almost invariably due to paralysis of the diaphragm, often with a terminal respiratory complication such as pneumonia. The great bulk of deaths occurs in the first week of illness with a few in the second, and scarcely any thereafter. The case fatality rate varies rather widely from epidemic to epidemic and locality to locality; an average figure is 20 per cent, with outside limits of 5 to 50 per cent. The rate is considerably higher in the case of children under one year and in elderly persons.

During the Second World War, British service personnel often suffered a high mortality rate in poliomyelitis outbreaks, for example, in Malta 11/57 men died (Bernstein, Clark, and Tunbridge, 1945), and in India the mortality rate rose to 37 per cent (McAlpine, 1945); a similar experience befell other troops

The mortality in bulbar cases varies from 60 to 80 per cent.

CLINICAL PATHOLOGY

The cerebrospinal fluid. Examination of the cerebrospinal fluid is of great diagnostic importance, particularly in the preparalytic stage Numerous authors have published the results of their examinations, in which there is almost complete unanimity (e g., Gay and Lucas, 1910, Draper and Peabody, 1912; Peabody, Draper, and Dochez, 1912; Fraser, 1913, Neal and Jones, 1916–19, Neal and Kahn, 1916–19, Zingher, 1917 *b*; Kolmer *et al*, 1917; Gordon, 1932; Drury and Sladden, 1939, Bernstein, Clark, and Tunbridge, 1945).

The changes begin in the preparalytic stage, and continue for a variable part of the paralytic period. The fluid is usually under slightly increased pressure, and hazy, after standing for some hours, a fibrin clot may form.

Increase in the cell count is one of the first manifestations of infection, beginning in the preparalytic stage, the greatest number of cells is to be found in the first week after the onset of paralysis. The count usually remains raised for 3 weeks after the onset of paralysis, and thereafter declines The total number of cells is seldc [illegible]
per ([illegible]

[illegible]

is more common in nonparalytic cases

The total protein becomes increased also but, unlike the cell count, is greater in the 3rd week than the 1st. The protein may become normal after 10 to 13 days, only to rise later (Bernstein, Clark, and Tunbridge, 1945). Some increase may still be present after 7 weeks. The colloidal gold curve is of the luetic or meningitic type, while the colloidal benzoin test is frequently positive (Kessel, Hoyt, and [illegible]

with or without complement, may be present (Kolmer *et al*, 1917). Spolverini (1946) found an increase in the ascorbic acid content.

It may be necessary to examine more than 1 sample, especially if the 1st is examined toward the end of the 2nd week, when the cell count may have fallen to normal, and the protein increase hardly have begun (Eyre-Brook, 1942).

Rehm (1939) examined cell deposits by histological methods, and found small acidophil granules in the nuclei of lymphocytes, which enlarged to form inclusions

It is not possible to relate clinical severity to the cell count or protein content.

The blood and urine Changes in the blood are not of such importance as those in the cerebrospinal fluid, but have been extensively studied by certain authors

usually after about a week, and by 4 to 6 weeks should be well advanced. It is generally believed that muscles still paralyzed after 6 months will never recover their function. Probably about one-third of all cases show some permanent paralysis and atrophy occurs with the production of characteristic deformities.

Some psychic disturbances may be found after convalescence, such as nervousness, anxiety, affections of sleep, and mental change (see Faber, 1933).

Persons who have suffered from poliomyelitis in early life are not more liable than others to contract encephalitis lethargica (Klimke, 1937).

THE KENNY CONCEPTION OF POLIOMYELITIS

Miss Kenny has advanced a very different conception of poliomyelitis from the orthodox one already outlined. In the orthodox view, damage to the anterior horn cell is the main factor, and produces flaccid paralysis of the muscle fibers innervated therefrom. Opposing muscles may contract and shorten, but are not recognized to be otherwise abnormal. A primary attack of virus on muscle has not been visualized. Miss Kenny's views of recent years have been published in various American sources (e.g., Kenny, 1943, 1943–4, 1944; Pohl and Kenny, 1943). Her theories appear to be as follows

1. Muscle spasm is present in every case of poliomyelitis, paralytic, and nonparalytic. The spasm is found in the muscles directly affected by the disease, and causes them to be painful and hyperirritable. This muscle spasm is made up of 2 phenomena, both resulting from hyperactivity and hyperirritability of the synaptic connections to the motor nerve cells in the cord, hypertonus of muscles, and the hyperirritable stretch reflex. Spasm is the primary lesion Although initiated in the CNS, it seems to be due to a direct effect on the muscle also.

2. The flaccid muscles are usually intrinsically sound, and can be restored to normal function by treatment The inability to contract these "paralyzed" muscles is due to "mental alienation" from the nervous system. A physiological "block" in the motor pathway is postulated, so that nervous impulses do not reach the anterior horn cells These nonfunctioning muscles can be contracted only on releasing spasm in its opponents, and on restoring the physiological continuity of the nerve conduction paths.

3 Incoordination of muscle action appears in the untreated case It is often found in a single muscle, and can be observed in reflex as well as voluntary motion. It is fundamentally a disorganization of muscle function on an involuntary level.

4 Paralysis due to destruction of the nerve cell is not nearly as common as is imagined Most supposed paralysis is due to untreated spasm and to disease in the dissociated muscle

5. Deformities resulting from orthodox methods of treatment are due to untreated muscle spasm

Miss Kenny's claim is that disturbance of physiological function of the nervous system is of more importance than structural damage to the nerve cells The disease is held to affect muscles directly as well as nerve cells Based on her theories, a system of treatment has been introduced, but it is not in our province to discuss the technique (see Pohl and Kenny, 1943)

According to Schwartz and Bouman (1942), spasm is a reflex phenomenon, and, therefore, absent in a completely paralyzed muscle Spasm is explicable in terms of a lesion at, or proximal to, the dendrite or cell body of the lower motor neuron, which partially isolates the lower neuron from the inhibition exerted by higher levels, spasticity and weakening are 2 separate phenomena, each dependent on disturbed functions of the anterior horn cells (Schwartz, Bouman, and Smith, 1944)

Kabat and Knapp (1944) state that spasm is of 2 types, either hypertonus, or due to the hyperirritable stretch reflex They suggest that spasm is the result of an increased discharge of impulses through the motor neurons, and claim that the pathological basis of spasm is a lesion of the internuncial neurons in the gray matter of the cord Chronaxia estimations showed that there is no correlation between spasm and damage to the anterior horn cells Other workers have referred to the question of spasm (Cole, Pohl, and Knapp, 1942, Fox and Spankus, 1945, Brainerd *et al*, 1945)

Paul (1938) recommends concentration of washings by freezing and drying in the presence of normal serum

Horstmann, Melnick, and Wenner (1946) recommend the following technique The throat swab is soaked in 10 per cent sterile horse serum in distilled water, and the throat rubbed vigorously. The patient then gargles on several occasions 50–75 c.c. of 10 per cent horse serum In young children a nasopharyngeal irrigation with 10 per cent horse serum can be used instead of gargling The swab material and garglings are then available for concentration by centrifugation or other means, prior to inoculation in monkeys

Howe and his coworkers employ 2 swabs, after use, the cotton portions are stored in 1 c c of sterile water on dry ice, and at a convenient time treated as follows (Howe *et al*, 1944, Howe, Bodian, and Wenner, 1945, Wenner, 1945) elution is performed in phosphate buffer at pH8, the cotton being squeezed out, the eluate is corrected to pH6, 20 per cent ether is added, and the fluid kept in the refrigerator till sterile, the ether is removed, and the fluid inoculated into both lateral thalami of a monkey

Washings may be collected by a mechanical irrigator (Atlas, 1947) Virus has been recovered from a mask (Ward and Walters, 1947)

Occurrence of virus. Virus has been recovered from nasopharyngeal washings on a number of occasions from preparalytic and from early spinal or bulbar paralytic cases (Du Bois, Neal, and Zingher, 1914, Amoss and Taylor, 1917; Flexner and Amoss, 1919, Paul, Salinger, and Trask, 1932, Paul and Trask, 1932, Paul, Trask, and Webster, 1935, Kramer *et al*, 1936, Stillerman and Brodie, 1937–8, Trask, Vignec, and Paul, 1938 *b*, Vignec, Paul, and Trask, 1938, Kramer, Hoskwith, and Grossman, 1939, Howe, Bodian, and Wenner, 1945, Melnick, Horstmann, and Ward, 1946, Howe and Bodian, 1947).

Nasal secretions and saliva usually fail to yield virus (Sabin, 1941 *b*), although Lucas and Osgood (1913) and Ward and Walters (1947) have isolated it from nasal secretion

tic cases (Du Bois, Neal, and Zingher, 1914; Amoss and Taylor, 1917, Flexner and Amoss, 1919). Sabin and Ward (1941 *d*) found virus in the pharyngeal wall but not in the nasal mucosa or salivary glands

It has been recovered also from tonsils and adenoids (Landsteiner, Levaditi, and Pastia, 1911 *b*, Flexner and Clark, 1911, Levaditi, Schmutz, and Willemin, 1930, Brodie, 1934–5 *d*, Kessel *et al*, 1941) In these cases of course, it is not possible to exclude contamination with pharyngeal secretion

The tracheal secretion of fatal cases may contain the virus (Kling, Wernstedt, and Pettersson, 1911–12)

Duration of infectivity Virus may persist in the nasopharynx for over 2 weeks from the time of onset of the preparalytic phase (Du Bois, Neal, and Zingher,

prove nega-
Horstmann,
on the 7th
carriage in

found may depend to a certain extent on the presence of virucidal properties in normal nasopharyngeal secretions (Amoss and Taylor, 1917). The active principle is water-soluble and thermolabile, and its potency may be diminished or lost in inflammatory states of the upper respiratory passages

Comparative examinations of stool and nasopharynx As will be described below, virus is constantly to be found in the stools of preparalytic and paralytic cases All the evidence suggests that virus is present for a very much longer time in the feces than in the throat For example, of 19 tests on pharyngeal swabs taken during the first week of illness, only 1 was found positive, of 16 tests on oropharyngeal washings, only 1 was positive, but of 10 inoculations of stools in the same period 7 were positive (Horstmann, Melnick, and Wenner, 1946).

(e.g., Gay and Lucas, 1910; Draper and Peabody, 1912; Peabody, Draper, and Dochez, 1912). From a study of these and other reports it appears that the commonest finding is of a leukocytosis up to about 25,000 per c.mm., in the paralytic stage. The differential count usually shows an approximate 10 to 15 per cent. increase of polynuclears with a relative lymphopenia. The sedimentation rate is usually normal (Fox and Evrard, 1946). A considerably increased excretion of coproporphyrin III in the urine was noted in a series of cases of acute poliomyelitis (Watson *et al.*, 1947).

Routine bacteriological examination. Examinations of the nose and throat, urine, and stools have never shown any significant abnormality; this observation has been reaffirmed by Toomey (1935 *a*). Convalescent sera are said to show an increase in *B. coli* and paratyphoid bacillus agglutinins (Toomey, 1931–2 *b*, 1934 *a*).

DISTRIBUTION OF VIRUS IN PREPARALYTIC AND PARALYTIC CASES

Virus must be constantly present in paralytic cases in the spinal cord, and probably also to a lesser extent in the base of the brain and in the higher centers. Although accurate titrations of virus have not been performed, it is probable that the quantity of virus in the cord, or bulb in bulbar cases, is considerably greater than in the brain. Virus cannot be demonstrated in the cord of every fatal case, perhaps for purely technical reasons, and it is easier to detect it in younger persons. Thus 71 per cent. of cords from patients under 16 years of age were positive, as compared with 31 per cent. from persons of 16 years and over (Kessel *et al.*, 1941).

The virus was rarely detected in the olfactory bulbs (Kessel *et al.*, 1941), and Sabin and Ward (1941 *d*) obtained negative results. It has been found in the intervertebral ganglia (Flexner, Clark, and Amoss, 1914 *b*), and abdominal sympathetic plexus (Sabin and Ward, 1941 *d*).

Most workers have failed to isolate virus from the cerebrospinal fluid in the preparalytic or paralytic stages (e.g., Leiner and Wiesner, 1909, Flexner and Clark, 1914, Amoss, 1928, Brodie and Wortis, 1934). Positive results have been claimed by Ceccaldi (1943).

It is not known whether virus is actually present in the central nervous system in the preparalytic period, but this appears highly likely, from comparison with monkey experiments (see Bodian, 1946, 1947).

Blood, Urine, Lymph Glands

Virus has not usually been detected in the blood (Flexner and Clark, 1911, Clark, Fraser, and Amoss, 1914, Amoss, 1928, Melnick, Horstmann, and Ward, 1946). Out of approximately 100 samples from patients suffering from abortive or paralytic attacks, a positive result was obtained in only 1 child, 6 hours after the onset of an abortive attack (Ward, Horstmann, and Melnick, 1946).

Virus has not been found in the urine (Sabin, 1941 *b*, Toomey, Tischer, and Takacs, 1943).

Virus has on a few occasions been isolated from mesenteric, inguinal, axillary, and cervical lymph nodes (Flexner and Lewis, 1910 *d*, Kling, 1937, 1938 *b*, Kling, Olin, and Gard, 1938, Wenner and Paul, 1947).

Investigation of a number of extraneural tissues proved negative (Kempf and Soule, 1940 *a*). It may be concluded that the thoracic and abdominal viscera only rarely contain virus.

Upper Respiratory Passages

Technique. Virus may be sought in throat washings or garglings, or in material removed by cotton wool swabs. Suspensions of tonsils can be examined, or of pharyngeal or nasal mucous membranes in fatal cases.

suspension separated into the lower aqueous fraction, the surface ether layer, and an intermediate layer of particulate "insoluble" material.

bacteriological examination The extract remained in the cold room until tests showed *that it was free from viable bacteria.* On removal from the cold room, the aqueous fraction was prepared for animal inoculation

During ether treatment, precautions were taken to reduce absorption by the extract of oxygen from the air The cysteine stool extract mixture showed reduction of thionin

fraction was
ie excess ether
ierature. If the
nder was pre-

pared for monkey inoculation, by the cerebral route

Gard's (1943 a) method The material was suspended in a suitable volume of 0.3 per cent. saline, and shaken with glass beads mechanically for 2-4 hours Further diluent was then added, and the mass extracted under mechanical stirring for 8 hours It was then *left to settle in the cold room overnight. The supernatant was siphoned off and passed* twice through the separator The clarified extract was mixed with 2/3 by volume of

suspended to the original volume in distilled water and run up and down to 16,000 r p m. The second supernatant was further fractionated in the centrifuge, alternating at 27,000 r p m. for the 70 minutes, and up and down to 16,000 r p m The Sharples type of instrument can be used if the deposit is sealed from the atmosphere (Melnick, 1946 *a*, 1947).

Silverberg (1945) has shown that virus can be sedimented at 18,000 r p m in 4 hours from stool suspension The sediment is treated with ether.

Centrifugation. Probably the best method of concentrating virus is by differential centrifugation Briefly, the method used by Melnick (1943 *a*) and coworkers (Melnick ... Ward, 1945) is as follows

at 3000 r p m, 18,000, 39,000, and finally at
ween centrifugations 1 and 2, and 3 and 4
is left in the cold for 16 hours, and then

inoculated intracerebrally.

Animal inoculation After treatment by one of the above methods, material is inoculated in susceptible monkeys intranasally, intraperitoneally, and, when the material is sterile, intracerebrally, or into the lumbar cord If possible the animal should be inoculated repeatedly, and 2 or more animals should be used (Howe and Bodian, 1942 *a*, 1944 *a*)

Bodian, 1939 *a*), in fact, this is perhaps one of the most satisfactory methods. These authors recommend the use of nembutal anesthesia, and 5 nasal inoculations, the animal being suspended head downward, for 15 minutes, the inoculation is made with a blunt needle *over the olfactory area*

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Intestinal Tract

Technique.

Virus may be sought in feces, or rectal washings, or in fatal cases in suspensions of the wall of the small or large intestine; at postmortem, colonic contents can be collected.

Prior to inoculation specimens can be preserved frozen, preferably in a carbon dioxide ice chest, infectivity is maintained for several months. Specimens can be mailed to the laboratory (Trask, Paul, and Vignac, 1940). If desired, specimens can be kept under ether in the ordinary refrigerator for at least 12 days (Kling *et al*, 1939 *a, b*). Usually on receipt in the laboratory the specimen is prepared for animal inoculation by one of the following methods

Desiccation from the frozen state can be used to concentrate stools (Kramer, Gilliam, and Molner, 1939).

Freezing and thawing. Fecal suspensions are strained through cheesecloth, subjected to one cycle of freezing and thawing, and lightly centrifuged. The supernatant is used for inoculation (Howe and Bodian, 1940 *a, b*).

Precipitation and dialysis Virus in aqueous suspensions of stool can be concentrated by precipitation with 50 per cent saturation of ammonium sulfate, and subsequent dialysis (Gard, 1940 *a*) Virus has been recovered also by precipitation of proteins at the isoelectric point, ether is added and inoculation carried out with the middle layer (Herriott and Francis, 1943).

Etherization Many workers have made use of ether as a means of destroying intestinal bacteria without affecting the virus (e g., Kling *et al*, 1939 *a, b;* Lépine, 1939, McClure, 1941 *a, c;* Paul and Trask, 1941).

Trask, Paul, and Vignec (1940) recommend breaking up the stool with 5-10 times its weight of cold distilled water. After a few hours in the cold, the supernatant is pipetted into flasks with glass beads, and 10-15 per cent. of ether by volume added. The flasks are shaken and kept in the cold overnight. The contents are pipetted into 50 c.c. tubes and centrifuged at 1000 r p.m. for 20-45 minutes. The middle layer is pipetted off, and held in the cold till inoculated If desired, fluid can be concentrated by lyophilization

A simple technique was used by Paul, Havens, and van Rooyen (1944). A 10 per cent suspension of 5 gm of stool is prepared in sterile distilled water. It is allowed to settle at room temperature, and the supernatant fluid is then divided into 2 parts. One part is kept at refrigerator temperature, and instilled (3 c.c.) into the nostrils of a monkey on 3-4 successive days The other part is centrifuged for 20 minutes at 2000 r p m, and 15 per cent. ether is added to the supernatant The etherized suspension is kept in the refrigerator for 48-72 hours, and 15-20 c c inoculated intra-abdominally in a monkey.

Fisher (1943) used the following method, working in Craigie's laboratory.

(*a*) Anesthetic ethyl ether (1 volume), taken from an unopened metal container kept at 5° C in the dark, was shaken with 2 changes of neutral distilled water (2 volumes) in a 250 c c separatory funnel for 5 minutes

(*b*) Cysteine hydrochloride was freshly prepared in a primary dilution of 1 50 The crystals were first dissolved in 1 c c of neutral distilled water and then 5 c c of 0 6 M Na_2HPO_4 were added The pH of this mixture was 4 3 The cysteine solution was then diluted to requisite volume by the addition of 0 06 M. sodium potassium phosphate buffer at pH 7 0 The resulting pH was 5 7

(*c*) A weighed amount of stool material was well triturated in a mortar with 0 85 per cent aqueous solution of sodium chloride, whic
the process, until a dilution of 1 10 was obtained T
in an angle head centrifuge for 30 minutes at 3,00
2/3 of the supernatant was removed to a separato
were added 5 c c of the cysteine hydrochloride solution to give a final dilution of the latter of 1 500 The pH of the stool extracts was remarkedly uniform being from 6 8 to 7.2, when tested colorimetrically with bromthymol blue However, on the addition of the cysteine solution the pH shifted to a range of from 6 0 to 6.4, at which pH the extract was treated with ether

(*d*) One volume of washed ether was added to 2 volumes of stool extract in the separatory funnel, and shaken for 5 minutes Following a 5 minute settling period, the

periods in the stool, and Lucas and Osgood (1913) found virus in the nasal secretion some months after an attack.

Carriage by contacts. It is now quite clear that poliomyelitis virus becomes widely distributed in the environment of abortive or paralytic cases of the disease Thus virus has on occasions been recovered from the nasopharynx of intimate contacts, especially members of the same family and playmates (Flexner, Clark, and Fraser, 1913, Kling and Pettersson, 1914, Melnick, Horstmann, and Ward, 1946) In another study, however, virus was not found in the nasopharynx, even though the stools were positive (Brown, Francis, and Pearson, 1945). Howe and Bodian (1947 *a*) recovered virus from the throats of at least 2/28 healthy children in distant contact with a source of infection Virus has more frequently been recovered from the stools of intimate contacts (Kramer, Gilliam, and Molner, 1939, Kessel *et al*, 1941, McClure, 1941 *c*, Piszczek *et al*, 1941; Top and Vaughan, 1941, Howitt, Buss, and Shaffrath, 1942, McClure and Langmuir, 1942; Langmuir, 1942; Brown, Francis, and Pearson, 1945, Gear, Yeo, and Mundel, 1945, Pearson and Rendtorff, 1945 *a, b*, Gear and Mundel, 1946, Casey *et al*, 1947).

While some of these contacts develop abortive, and a few paralytic, poliomyelitis, many remain in apparent health. It can be said, therefore, that "healthy" carriers of poliomyelitis definitely exist These persons probably remain carriers for only a short while, yet may disseminate the infection in the meantime, they probably carry the virus for the same length of time as do cases. For example, Piszczek *et al.* (1941) found virus in the stool 2 months after contact

The majority of positive isolations of virus in contacts has been from children, who seem to be more liable to "pick up" the virus than adults. Even infants may be "healthy" carriers (Kramer, Gilliam, and Molner, 1939, Pearson and Rendtorff, 1945 *b*) Nevertheless, various workers have detected virus in the stools of adults in contact with cases Thus virus has been isolated from nurses (Kramer, Gilliam, and Molner, 1939, Landsberg, 1945). Langmuir (1942) recovered virus from the stools of 4/8 adults who were intimate contacts of the cases Pearson *et al.* (1945) found virus to be harbored by adults in 5/6 households where a case occurred

Carriage by noncontacts The available evidence shows that the virus enjoys a limited distribution in close relation to cases of the disease. It is found only rarely in the secretions of persons with no known contact with poliomyelitis Thus Pearson and Rendtorff (1945 *a*) examined the pooled feces of over 100 persons in a village The only positive was the 6-year-old son of a patient. In another outbreak, a larger number of stools was examined, and again the positives were found to have some personal association with cases, with one exception (1945 *b*) Similar negative results with the stools of noncontacts have been recorded by others (Trask, Paul, and Vignec, 1940, Piszczek *et al*, 1941, McClure and Langmuir, 1942). The stools of persons suffering from other diseases have also been examined as controls, and found negative (*Paul, Havens, and van Rooyen, 1944*).

However, certain observations show that on occasions the virus may be recovered from persons with no known contact with cases of poliomyelitis Thus virus [illegible]

[illegible] posure

[illegible] known

[illegible] groups

[illegible] 5/136

persons harbored virus either in the tonsils or stool. These latter findings suggest that perhaps the virus enjoys a wider distribution than has been believed hitherto

"Incubating" or "precocious" carriers Persons harboring virus in the nasopharynx may later develop symptoms of poliomyelitis (Taylor and Amoss, 1917). Virus was detected in the stool of a boy who 12 days later developed clinical poliomyelitis (Gear and Mundel, 1946), and of another person who became ill 19 days later (Brown, Francis, and Pearson, 1945) Obviously these persons may infect other persons during their incubation period (see Gear and Mundel, 1946).

paralytic, excretes virus in the feces for at least a brief period, and numbers of workers have isolated virus from such persons (e g, Kling, Wernstedt, and Pettersson, 1911–12; Harmon and Levine, 1936–7; Kling, 1938 *b*, Trask, Vignec, and Paul, 1938 *a*, *b*; Kling *et al.*, 1939 *a*, *b*, *Kramer, Hoskwith, and Grossman, 1939*, Lépine and Sédallian, 1939, Toomey, 1939 *c*, Howe and Bodian, 1940 *a*, 1942 *a*, Trask, Paul, and Vignec, 1940; Kempf and Soule, 1941; Kessel *et al*, 1941; McClure, 1941 *a*, *c*, Paul and Trask, 1941; Sabin, 1941 *b*, Sabin and Ward, 1941 *d*, *e*; Howitt, Buss, and Shaffrath, 1942, McClure and Langmuir, 1942; Paul, Havens, and van Rooyen, 1944; Silverberg, 1945; Gear and Mundel, 1946; Horstmann, Melnick and Wenner, 1946, Melnick, Horstmann and Ward, 1946, Ward, Horstmann and Melnick, 1946). Melnick (1947) has calculated that a 100 gm. stool may contain 1000–*10,000 monkey infectious doses*.

Virus has been found in the wall of the ileum, and less commonly of the descending colon (Sabin and Ward, 1941 *d*). Wenner and Paul (1947) isolated virus from the duodenal wall in 2 cases.

Effect of age. Virus has been found by some workers more readily in the stools of patients under 8–16, than in those of older persons (Trask, Paul, and Vignec, 1940, Kessel *et al.*, 1941, Sabin, 1941 *b*, Sabin and Ward, 1941 *e*). Horstmann, Ward, and Melnick (1944, 1946) found, however, that age played no part. Even infants (under 2) may excrete virus (McClure, 1941 *c*).

Duration of infectivity.

Virus has been found in the stools of an abortive case 2 days after onset (Ward, Horstmann, and Melnick, 1946), and on the day of onset of a paralytic case (Zintek, 1947). During the first week it is probable that all cases of abortive and paralytic poliomyelitis excrete virus, thereafter the number excreting steadily falls For example, *Horstmann, Ward, and Melnick (1944, 1946) studied the duration of* excretion of virus in 46 paralytic and 15 abortive cases, and found the following percentages of excretors in the various weeks first 2 weeks, 60–70 per cent, 3rd and 4th weeks, 50 per cent, 5th and 6th weeks, 27 per cent.; 7th and 8th weeks, 13 per cent, between the 9th and 24th weeks, virus was detected in only 1 specimen, at the 12th week

Other records of prolonged excretion are as follows. 50 days (Howitt, Buss, and Shaffrath, 1942), 2 months (Kessel *et al*, 1941), 123 days (Lépine, Sédallian, *and Sautter, 1939*)

We may conclude that virus can be found in the stools of abortive and paralytic cases equally, from shortly after onset until up to 3 months later, the majority ceasing to excrete in 1–2 months There is no evidence that virus is excreted for periods as long as a year or more (see, e g, Howitt, Buss, and Shaffrath, 1942)

Source of virus It has been suggested that virus in the stool is only derived from the nasopharynx, where it is present for a few days at the onset of the disease As virus has been isolated from the wall of the gut, and as it persists in the stool *long after it has disappeared from nasopharyngeal washings*, this does not appear very likely (Sabin, 1941 *b*, Sabin and Ward, 1941 *e*) Howe and Bodian (1942 *a*) suggest that virus is derived either from nerve cells of the intramural plexuses, from lymphocytes passing through the wall of the gut, or from epithelial cells of the mucosa It appears possible that virus may actually proliferate in epithelial cells of the intestine (see Evans and Green, 1947)

CARRIERS OF POLIOMYELITIS VIRUS

Convalescent carriers It is not thought that true convalescent carriers exist at all commonly, as all the evidence suggests that virus is present in the nasopharynx for only a few days, and in the stools for some weeks It is possible, however, that a very small percentage of persons may excrete virus intermittently over long

afferent systems of the 5th, 7th, 9th, and 10th cranial nerves, the first neuron is in the peripheral ganglia, while the first synapse is in the pons or medulla. With the sympathetic system the first neuron and synapse occur in the ganglion, while in the vagal efferent (parasympathetic) system the first neuron and synapse are in the intramural microganglia, very close to the mucous membrane. The best method of detecting the portal of entry, therefore, is to examine the site of the first neuron, in olfactory bulb, brain, or ganglion It must, however, be realized that changes in

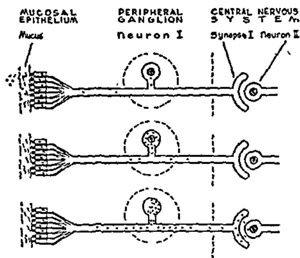


Fig 41 Route of invasion of poliomyelitis virus Schematic representation of invasion of a unipolar neuron with branched axon (e g, a trigeminal afferent unit of the gasserian ganglion) by virus particles (solid dots) through the epithelial surface, with subsequent central spread of infection Virus particles are caught in the mucus covering the epithelium, enter the superficial arborizations of the axon, course through the axon into the nerve cell (Neuron I) where they multiply, set up the primary lesion, then spread centrally, break through the synaptic membrane, and infect Neuron II Logically they may be expected also to course outward again to the surface, as in the lower figure, thus possibly accounting for the appearance of virus on the surface during the early clinical manifestations of the disease

(Reproduced from *J. exp. Med*, 1946, 83, 329)

ganglia may be produced not only by centripetal spread, when they are found early, but also by centrifugal spread at a later stage of infection (see also Bodian and Howe, below).

Faber and Silverberg visualized the invasive process as proceeding in steps (1) invasion of peripheral fibers into regional
tioned), (2) invasion of connecting center in
via short connecting neurons, (4) invasion of
and central sites of infection, presumably by
a detailed histological study of 8 fatal cases
their results as follows (*a*) the trigeminal (5th cranial) nerve afferent system (nose, mouth, pharynx) was very frequently involved (*b*) The visceral afferent

PATHOGENESIS OF HUMAN POLIOMYELITIS

Experimental

Theories of the pathogenesis of human poliomyelitis have resulted very largely from experimental work carried out on monkeys, rather than on observations in the patient. It is probable that in certain respects, work on monkeys has proved definitely misleading, particularly as suggesting that the olfactory route is commonly followed in the human subject. The main points that have been shown experimentally will be summed up here, but a fuller discussion will be found in the section on the pathogenesis of experimental poliomyelitis (see p. 988), and elsewhere in the sections devoted to the virus and its properties.

(*a*) The monkey can be readily infected by nasal instillation, and the virus spreads along the olfactory nerve fibers After a few days, both virus and histological changes can be demonstrated in the olfactory bulbs. Thereafter, the virus spreads to other parts of the central nervous system, and can be demonstrated successively in the thalamus, hypothalamus, medulla, cervical and lumbar cords (see p. 976).

(*b*) Although animals can be infected intrathecally, the cerebrospinal fluid is not held to play any part in the primary dissemination of the pathogenic agent.

(*c*) The monkey can be infected only with difficulty by the blood stream, and the blood is not usually responsible for the spread of the virus when it is injected by other routes

(*d*) Although earlier workers failed to infect by the gastro-intestinal tract, Toomey and others have recently succeeded in infecting by ingestion, pharyngeal swabbing, or direct injection.

In brief, the position on the experimental side is that virus spreads by axonal routes from the site of inoculation to the central nervous system. Arrived at the central nervous system, the primary attack of the virus is on the nerve cell, and there is no evidence that the degeneration which these cells undergo is in any way secondary to meningitis, or to interstitial lesions. The virus is present in the central nervous system from the beginning, and there is no stage of general infection with the presence of virus in the blood.

Portals of Entry and Spread of the Virus in Man

It is uncertain as to how poliomyelitis virus enters the human body. This question is intimately linked with epidemiology, and it is of considerable importance to discuss in some detail the various possible portals. This question has been studied for many years by Faber, whose opinions must be regarded as authoritative According to his latest views, the following possible portals of entry must be considered (Faber and Silverberg, 1946) the olfactory area, the nose, mouth, and pharynx, the tracheobronchial tree, the esophagus, the stomach and intestine proximal to descending colon, the distal colon, sigmoid, and rectum. (See Figs 41 and 42, from his paper) Faber and Silverberg presume that virus entering at any one of these sites invades the superficially situated axons of a nerve cell, and passes along the fibers to the first neuron (nerve cell), where it multiplies It is not thought likely that nerves without a superficial connection are likely to transmit the virus, with the exception of motor nerves in the pharynx after tonsillectomy The first neuron may have other axons passing directly to the brain where the first synapse occurs In other cases the first synapse occurs at the site of the first neuron. It is probable that the synapse represents a definite barrier to the progress of the virus, so that the synapse may show early lesions associated with invasion

In the case of the olfactory system, the first neuron is in the nasal mucous membrane, and the first synapse in the olfactory bulb In the mesencephalic trigeminal system the first neuron and synapse are in the midbrain In the case of the

in the mucous surfaces can, if exposed, convey virus centrally. There was evidence of entry through the nose, mouth, or pharynx in nearly all cases, and through the upper tracheobronchial tree or esophagus in several

CNS They suggested that ganglionic lesions could be caused by centrifugal, rather than centripetal, spread Another complication arises from the probability that virus can spread along nerves without producing changes in ganglia Faber and Silverberg found lesions in a number of ganglia and therefore postulated several portals of entry in a single patient Bodian and Howe mention that the 5th, 7th, and 9th cranial nerves serving the oropharynx, and the 10th nerve supplying the lower alimentary tract are the most likely routes of passage of virus to the CNS They conclude that the specificity of lesions in the sympathetic ganglia has not been established, and that the presence of lesions in the sensory ganglia does not reveal whether the virus reached the ganglia from the periphery or center, except in very early cases

It now falls to record a number of other observations regarding the rôle of the various possible portals of entry.

1. *The nose.* Virus has quite frequently been recovered from throat washings in abortive and paralytic poliomyelitis, and has been recovered from the tonsil, the nasal secretion seems to be less regularly infected (see p 928). Virus can usually be found in the nasopharynx in the first few days of the illness, and has been found in close contacts and "incubating carriers" Such persons must, therefore, exhale droplets of secretion infected with virus The symptoms of the illness of infection suggest upper respiratory involvement. Further, poliomyelitis seems sometimes definitely to be precipitated by respiratory infection. In some cases, the first symptoms of the declared disease are respiratory.

There is, therefore, evidence suggesting that the olfactory nerves must be seriously considered as a portal of entry The olfactory nerve endings lie exposed in the upper part of the nasal cavity, and virus present in the nose might readily come in contact with these structures The further spread of the virus would presumably be by the olfactory bulbs, olfactory bundle, fibers passing to the thalamus and tegmentum, and from the midbrain, by the spinothalamic tract to the posterior horn, from the posterior horn, fibers connect with the anterior horn (Harbitz and Scheel, 1907 *a*, *b*, Flexner, 1915, Faber, 1933) It has been mentioned that the clinical features of Phase 1 and 2 may be explicable by successive involvement of the *thalamus and hypothalamus and the sensory column* (Faber, see p 923).

Against the olfactory route as a common portal of entry, however, are certain important observations Thus, histological changes occur only rarely in the olfactory bulbs (see 942), and virus does not appear to be regularly present in these structures (see p 928). Further, trials of chemical substances intended to "block" the spread of infection by the exposed olfactory nerves have been a failure (see p 966)

A few years ago the balance of opinion was in favor of accepting the olfactory route as the common portal of entry (see, e g, Flexner, 1931 *b*), but few now hold this view. Faber and Silverberg (see above) in histological studies found no support for the concept of entry by olfactory nerves, but concluded that virus commonly entered through the nose and nasopharynx, involving the trigeminal afferent system, and the visceral afferent system of the 9th and 10th cranial nerves

system of the 9th and 10th cranial nerves (nose, mouth, pharynx, esophagus and bronchi, stomach and intestines) was fairly commonly involved. (*c*) The gustatory system (7th, 9th, and 10th cranial nerves) was occasionally involved. (*d*) The sympathetic system was occasionally involved (i e, pharynx, bronchi, esophagus, intestines). (*e*) The vagal efferent or parasympathetic system and the olfactory system were not involved.

In general, the evidence of penetration through the upper alimentary and

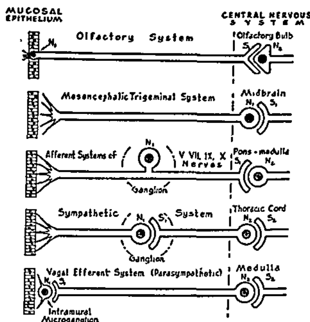


FIG 42 Route of invasion of poliomyelitis virus Schematic representation of the various potential nerve pathways of infection, showing the different arrangements of neurons (N_1, N_2) and synapses (S_1, S_2) in relation to the mucous surfaces, peripheral ganglia, and central nervous system Note unique position *inside the central nervous system* of the primary neuron of the mesencephalic trigeminal system Its peripheral endings are, however, deep from the surface, making them relatively inaccessible to surface virus except under special circumstances, such as are discussed in the text

(Reproduced from *J exp Med*, 1946, *83*, 329)

respiratory tracts was more obvious than through the lower alimentary tract Evidence for intestinal entry through the sympathetic was ambiguous, but there was little evidence of infection ascending to the CNS by this route The pharynx appeared to be a specially favorable site for the primary penetration of the virus Primary invasion through the sympathetic system results in initial involvement of the CNS at the spinal level, invasion through the other channels results in initial involvement of the CNS at the brain stem level In neither instance does the level of this initial involvement necessarily determine the site of initial paralysis

They concluded that there appears to be no single portal of entry in human poliomyelitis, on the contrary, their evidence suggested a variety. The earlier concept of an exclusive channel for all cases, either olfactory or intestinal, is, therefore, contradicted. Apparently almost any nerve system with peripheral endings

in the mucous surfaces can, if exposed, convey virus centrally. There was evidence of entry through the nose, mouth, or pharynx in nearly all cases, and through the upper tracheobronchial tree or esophagus in several

It is uncertain how much value can be attached to deductions based on changes in ganglia, and Bodian and Howe (1947) have come to somewhat different conclusions from those of Faber and Silverberg. They found no lesions that could be considered specific in complete serial sections of 16 pairs of celiac ganglia, and 11 pairs of stellate ganglia from fatal human cases. There were abundant lesions, however, in some of the trigeminal and spinal sensory ganglia. The intensity of the lesions coincided relatively well with the intensity of the lesions in the adjacent CNS. They suggested that ganglionic lesions could be caused by centrifugal, rather than centripetal, spread Another complication arises from the probability that virus can spread along nerves without producing changes in ganglia. Faber and Silverberg found lesions in a number of ganglia and therefore postulated several portals of entry in a single patient Bodian and Howe mention that the 5th, 7th, and 9th cranial nerves serving the oropharynx, and the 10th nerve supplying the lower alimentary tract are the most likely routes of passage of virus to the CNS They conclude that the specificity of lesions in the sympathetic ganglia has not been established, and that the presence of lesions in the sensory ganglia does not reveal whether the virus reached the ganglia from the periphery or center, except in very early cases

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Other histological studies have failed to incriminate the olfactory portal (Swan, 1939, Robertson, 1940, Howe and Bodian, 1941 *d*, 1942 *a*).

2. *Mouth and pharynx.* Numerous cases of bulbar poliomyelitis have followed tonsillectomy (see p. 920). It is probable also that trauma associated with the cutting of teeth may allow the entrance of virus into nerve fibers It has been suggested also from clinical observation and experiments in monkeys that pulpal exposure may act as a predisposing factor (Aisenberg, 1946; Aisenberg and Grubb, 1943, 1945) Virus has been isolated from the tonsil and nasopharyngeal secretions in healthy contacts, incubating carriers, and cases of paralytic or abortive infection There is every reason to postulate, therefore, that the mouth and pharynx may be a common portal of entry in ordinary cases of poliomyelitis, and Faber and Silverberg found evidence of entry through the nose, mouth, or pharynx in nearly all cases. These workers suggested that the nasopharynx and oropharynx are probably *the most important portals of entry, and are infected either by mucous* drainage from the nose or by material entering the mouth. Virus spreads back to the CNS via the trigeminal afferent, 9th and 10th afferent, or less commonly the gustatory system Other workers have also obtained evidence by histological examination that cranial nerves connected with the pharynx may act as portals of entry in ordinary cases (Robertson, 1940; Howe and Bodian, 1941 *d*, 1942 *a*, Sabin, 1941 *a*, 1942).

In cases of primary bulbar poliomyelitis, not connected with tonsillectomy, it is probable that the virus spreads to the medulla by invasion along the 5th, 7th, 9th, and 10th cranial nerves (Sabin, 1942) and not by the sympathetic pathway.

3. *Tracheobronchial tree* Virus has been recovered from tracheal exudate, and apart from this the common presence of virus in the upper respiratory passages suggests the possibility of invasion through the mucosa of the trachea and bronchi The only observations are those of Faber and Silverberg who concluded that several cases showed evidence of entry through the upper tracheobronchial tree (as well as by the nose, mouth, and pharynx). The paths involved are the visceral afferent system of the 9th and 10th nerves, or the sympathetic system

4 *The gastro-intestinal tract* The gastro-intestinal symptoms of the preparalytic stage, and the presence of lesions of the follicles and Peyer's patches, led many authors to suggest that the virus enters the body by the gastro-intestinal tract (e g, Harbitz and Scheel, 1907 *a*, *b*, Wickman, 1907, Krause, 1909, Dahm, 1909, Burrows, 1920, 1931, Kling, Levaditi, and Lépine, 1929, Toomey, 1931–2 *c*, 1933, 1934 *a*, Kling, 1937, Gard, 1937, 1938).

With the knowledge of the relative ease with which virus can be isolated from the feces of cases and contacts, there is every justification for this suggestion Experiments conducted *in vitro* indicated that at high levels of acidity, e g, *pH 1 0*, virus is completely inactivated in 5 minutes, at pH 2 0 inactivation is not complete for 4 hours Conditions of high acidity as occur after ingestion of carbohydrate or meat, therefore, favor inactivation Milk would tend to prevent inactivation. It appears, therefore, that ingested virus may not uncommonly reach the intestine (Faber and Dong, 1946). It has been held that virus travels to the cord by the sympathetic system, and Toomey has followed up this suggestion of older workers (1936 *f*)

One of Toomey's main points is that the virus has a special affinity for gray fibers Medullated fibers contain very much more cholesterol than gray fibers Accordingly, as it has been found that cholesterol inactivates the virus *in vitro*, he has postulated that medullated fibers offer a certain resistance to the spread of the virus, but on occasions this may be overcome Toomey suggests that the virus of poliomyelitis enters the body through naked nerve endings Such a distribution of fibers exists in the nose, the gut, and in the terminal ramifications of the chorda tympani Toomey believes that the observed facts of poliomyelitis are best explained

by postulating entry through the gut In certain cases, however, virus may enter the body through nerve endings in the mouth, and give bulbar symptoms.

The virus is said to spread from the gastro-intestinal tract by the sympathetic gray fibers and somatic nerves and, as the virus has an affinity for gray fibers one would expect that the parts of the body to be most commonly involved would be the legs, because between the 3rd lumbar and 2nd sacral segments only gray fiber connections appear Elsewhere, the white rami communicantes are thought to hinder the spread of virus The virus may spread up the sympathetic chain to the cervical area, where there is a similar absence of white rami communicantes, and cause paralysis of the upper extremities

As evidence of this affection of the sympathetic fibers, Toomey mentions that the bladder is often paralyzed before somatic paralyses appear Further, he relates the various reflex changes, and the results of pilocarpine injection (see above), to involvement of the sympathetic fibers.

Of recent years histological examination of the nervous system has been carried out by various workers, and has suggested to them that in many cases the virus appears to reach the cord from the bowel by nerve paths (Howe and Bodian, 1941 *d*, 1942 *a*, Sabin, 1941 *a*, 1942; Faber and Silverberg, 1946).

Sabin suggests that virus enters the body by the mouth and establishes itself in the alimentary tract, where it multiplies Invasion of the CNS occurs along 2 paths One path passes to the medulla by way of the cranial nerves or by way of the parasympathetic system of the vagus The other path leads to the spinal cord along visceral afferent fibers through spinal ganglia, an alternative route is by visceral efferent fibers and the sympathetic chain.

Faber and Silverberg concluded that the evidence of penetration through the upper alimentary tract (pharynx and esophagus) was more obvious than through the lower tract There was little evidence of infection ascending to the CNS by the sympathetic system.

Although virus would appear to proliferate in the wall of the small intestine, the evidence suggests that invasion of the CNS more usually occurs from the upper alimentary tract, especially the pharynx It is possible, however, that a secondary invasion of virus may occur from the intestines, explaining the relative frequency with which lesions are found in the celiac ganglia (Faber and Dong, 1946)

There is no evidence to suggest that virus may invade through the colon and rectum.

5 *The skin* The rôle of the skin has received relatively little attention It is known that poliomyelitis has followed the subcutaneous inoculation of vaccines If mosquitoes or biting flies play any part in transmitting the disease, virus would require to be introduced into or under the skin

Wenner and Paul (1947) studied a fatal case in a technician, where the portal of entry may possibly have been a scratch on the wrist which was later soiled with virus

Possible Alternative Means of Dissemination of Virus

There is no doubt that poliomyelitis virus in man and monkey is essentially neurotropic, and invades the CNS from the portal of entry by nerve paths This method of spread has been suggested in man by histological observations, and in monkeys by various experiments. One must, however, consider certain alternative methods of invasion

1 *Cerebrospinal fluid.* It has been suggested that virus enters the cerebrospinal fluid, probably from the olfactory perineural spaces, and is disseminated thereby to the cord and other parts of the central nervous system (see Amoss, 1928) However, as the virus has not been commonly isolated from the cerebrospinal fluid, it is not easy to justify this theory.

2. *The blood* Although widely held at the beginning of the century (e g., by Lovett and Lucas, 1908, also see Wayson, 1918 *a*), this theory was mainly formulated by Draper (1917), who postulated a stage of generalized infection, with virus in the blood. It was thought that the virus caused injury to the meningeal vessels and plexuses, finally producing a generalized infection of the central nervous system. Virus has, however, only very rarely been demonstrated in the blood of human cases. The pathological lesions, moreover, do not suggest any such method of spread.

3. *The lymphatic system.* It has been known for some time that the lymphoid structures are involved in poliomyelitis (e.g , Müller, 1910, Peabody, Draper, and Dochez, 1912; Abrahamson, 1916–19 *a*, *b*). For example, the intestinal follicles are enlarged, the Peyer's patches prominent, and there is enlargement of the spleen and mesenteric lymph nodes In one case, Flexner and Lewis (1910 *d*) isolated the virus from a mesenteric lymph node (as did Kling, 1937). There is also enlargement of many other groups of lymph glands, such as the substernal and bronchial, cervical, axillary, and inguinal The tonsils are enlarged, and may contain the virus (see p. 929)

Burrows (1920, 1931), in particular, has stressed the importance of the lymphatic system and has even gone so far as to call poliomyelitis a lymphatic disease He suggests that the virus enters the body by the lymphatics of the gastro-intestinal tract, and then generalizes. He believes that perivascular lesions are primary and nerve cell lesions follow Faber (1933) suggests that the lymphatic hyperplasia is merely compensatory, to supply the lymphocytes needed both in the cord and the cerebrospinal fluid.

The Primary Tissue Reaction in Human Poliomyelitis

For many years controversy turned on what constitutes the primary reaction to the virus of poliomyelitis That nerve cell destruction is one of the most characteristic histological features of the disease was admitted by all, but the etiology of this lesion caused disagreement. One school, following the original lead of Charcot and Joffroy (1870), suggested the nerve cell lesion to be primary and independent of meningeal or interstitial inflammation (e g., Rissler, 1888, Abrahamson, 1916–19 *b*, Faber, 1933, Korn [illegible]). The other school held the nerve cell lesion to b[illegible] or perivascular cellular infiltration (e g., Roger [illegible] 1907, 1913, Harbitz and Scheel, 1907 *a*, *b*, Peabody, Draper, and Dochez, 1912) The question is difficult to solve from examinations of human autopsy material only, for at the time of death, lesions are so far advanced that it is not possible to state whether neuronal or interstitial change is the primary. But the work of Hurst and of Fairbrother (*vide infra*) on the pathogenesis and histology of experimental poliomyelitis, has shown conclusively that the nerve cell lesion is primary and quite independent of interstitial changes In our opinion, this is the only view that can be taken of the primary tissue reaction in human poliomyelitis, and is supported by later observations (Sabin, 1942, Elliott, 1945, Bodian, 1947)

PATHOLOGY

Numerous workers in many parts of the globe have reported the results of their pathological examinations, and the resulting picture is extremely uniform (e g, Forssner and Sjovall, 1907, Harbitz and Scheel, 1907 *a*, *b*, Wickman, 1907, Cadwalader, 1908, Peabody, Draper, and Dochez, 1912, Muller, 1910, Abrahamson, 1916–19 *b*, Burrows, 1920, 1931, McIntosh and Scarff, 1928, Marinesco, Manicatide, and Draganesco, 1929, Cappell, 1930–1, Wolf and Orton, 1932, Spielmeyer, 1932, Horányi-Hechst, 1935, Harmon and Levine, 1936–7, Peters, 1938, Swan, 1939, Sabin, 1942, Peers, 1943, van Rooyen and Morgan, 1943, Elliott, 1945, 1947, Faber and Silverberg, 1946, Luhan, 1946, Bodian, 1947, Scheinker, 1947).

Central Nervous System

To the naked eye, the brain and cord appear superficially congested. On section the cord is edematous, and the gray matter congested and sharply demarcated from the white

Types of lesion.

1. *Changes in the nerve cells.* It has been mentioned that nerve cell lesions are now regarded as primary, not secondary They are present even in the pre-paralytic stage, but do not attain any great intensity until the paralytic stage.

In the cord, the cells mainly affected are those of the anterior horn, although numerous cells in the posterior horn and ganglia are involved as well The changes vary from simple chromatolysis to complete destruction, cells which are no longer viable undergo neuronophagia, microglia and polymorphs removing all traces Cells which are not severely damaged, perhaps by edema only, presumably recover their function, thus accounting for the return of power to originally paralyzed limbs Sabin (1942) points out that the segmental distribution of the lesions may be so irregular as not to affect the major innervation of a given muscle After the lapse of time, if there has been much destruction of nerve cells, the horn becomes shrunken and atrophied The former site of the cells is occupied by glial tissue Necrosis of nerve cells occurs in the cerebral cortex, but is said to be confined to the Betz cells of the area giganto-pyramidalis (Swan, 1939).

Elliott (1945, 1947) has examined the nerve cells in the spinal cord in chronic and acute cases Lesions appeared to begin in the dorsal and medial nuclei and spread ventrolaterally. The degree of involvement varied from small localized foci to almost complete loss He suggested that the differential infection of nuclei was due not to their proximity to a source of infection, nor to their intrinsic suscep [illegible]

2 [illegible]

lesions [illegible]

(Bonh [illegible]

bodies do not show advanced degenerative change. The bodies are small eosinophilic granules measuring up to 3 to 5 μ in diameter The affected nucleus usually shows margination of the basichromatin Wolf and Orton have found similar bodies in a number of other diseases, and they would appear to resemble other well-known intranuclear inclusions, especially those of yellow and Rift Valley fevers.

3 *Perivascular infiltration* The vessels in the parenchyma of the cord, and to a lesser extent elsewhere, show "cuffing" The vessels mainly affected are those near the anterior and posterior median fissures and at the tips of the horns The predominant infiltrating cell is the lymphocyte, but some polymorphs and plasma cells are often seen.

4 *Focal and diffuse infiltrations.* The substance of the cord and certain other parts of the central nervous system show focal and more diffuse infiltration with lymphocytes, polymorphs, and particularly with microglia This microglial proliferation is a marked feature, and it is only comparatively recently that the true nature of these cells has been realized Older authors alluded to them as "polyblasts," "elongated glial cells," and by other names, and suggested their derivation from the lymphatic system.

[illegible]

mar [illegible]

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been previously injected intrathecally (Burrows, 1920).

Distribution of the lesions.

The regions most frequently involved are as follows, according to Sabin (1942), Bodian (1947), and others.

1. *The cord.* The lesions are most marked in the cervical and lumbar enlargements, and occur mainly in the anterior horn, although the posterior and intermediate horns are also involved. In acute bulbar cases, there may be no changes in the anterior horns, with some neuronophagia in less acute cases.

2. *The ganglia and nerves.* Cellular infiltrations occur in the gasserian and posterior root ganglia, and in the posterior and anterior nerve roots There is usually some loss of sensory neurons in the posterior root ganglia Marinesco and Draganesco (1932) found the ganglionic lesions to be as prominent as those of rabies.

3. *The medulla* In spinal cases changes occur in the vestibular nuclei, reticular formation, and the nuclei of various cranial nerves. In acute bulbar cases there is a marked incidence of neuronal lesions in the region of the nucleus ambiguus, dorsal motor nuclei of the vagus and nucleus solitarius, lesions become fewer as the cord is traced down (see also Goodpasture, 1925).

4. *The cerebellum* shows lesions in the roof nuclei and vermis (see also Burrows, 1920).

5. *The midbrain* shows changes in the periaqueductal gray matter, the tectum and tegmentum (see also Harbitz and Scheel, 1907 *a*, *b*; Burrows, 1920).

6. *The basal ganglia.* The hypothalamus and thalamus show cellular infiltrations

7. *The globus pallidus* shows lesions.

8 *The cortex* does not usually show much change, but lesions are concentrated in Brodmann's area No 4. Luhan (1946) has drawn attention to the frequency of changes in the motor area

9. *The olfactory bulbs.* [illegible]
tions been demonstrated,
and Smith, 1934, Harmon
De Groat, 1938, Swan, 1939, Robertson, 1940, Sabin, 1940, Luhan, 1946)

10. *Distribution of brain lesions.* Bodian (1947) found in a series of 24 cases that the centers most frequently severely damaged were the cerebellar roof nuclei, vestibular nuclei, and the reticular formation. Severe lesions in this group may superimpose on muscles weakened by imperfect spinal innervation, varying degrees of altered suprasegmental control, clinically appearing as varying degrees of hyper- or hypo-tonia, ataxia, tremor, and weakness

He found brain lesions in all 24 cases, most severe in the brain stem and not the cortex. Great variations occurred in the severity of the lesions in the brain, but the same centers were generally involved in all cases

Residual lesions.

Full descriptions of the residual lesions have seldom been published, although a few observations have been made (see International Committee, 1932) Peers (1943) has given the following account of 3 cases surviving for 7, 15, and 18½ weeks from the time of onset In the cerebral cortex there were perivascular collars of lymphoid cells and interstitial foci of microglia and astrocytes *confined to the paracentral lobules.* There was probably some diminution in the Betz cells One case showed thromboses in small vessels, with patches of recent infarction at the vertex The basal ganglia and thalami showed only minimal lesions. In the midbrain the substantia nigra was severely affected Perivascular infiltration and collections of astrocytes and microglia were found in the lateral tegmental and red nuclei There was a loss of nerve cells in Deiter's nuclei and the motor nuclei of the 5th and 7th nerves.

The longer the convalescence, the less was the perivascular infiltration, and the greater the gliosis

In the cerebellum there were some cell loss and astrocytic hyperplasia in the tectal nuclei and cortex of the vermis

In the medulla there were cell loss and scarring in the reticular substance, and a few dead nerve cells could be seen

The cord showed an almost complete loss of nerve cells through the whole length of the anterior gray substance. The lateral horns were comparatively spared. The anterior horns showed a replacement gliosis. A few necrotic nerve cells were still seen at 7 weeks. The white matter showed a mild diffuse demyelination of most of the ventral and lateral columns, excepting the pyramidal tracts. In the posterior columns, the partial demyelination was confined to the region of Schultze's tracts. Much of this degeneration was due to factors other than loss of the known cells of origin.

The anterior nerve roots showed severe degeneration consequent upon loss of the anterior horn cells Almost all the motor fibers had disappeared In contrast, the fine myelinated efferent sympathetic fibers were chiefly spared. At 7 weeks the nerve tubes contained many fat-laden phagocytes, which had disappeared by [illegible] ganglia there were a very [illegible] only a few cells had disap- [illegible] rs. Flynn (1946) examined [illegible] of the anterior and lateral horns were replaced by granulation tissue The muscles showed an extreme degree of atrophy of degeneration.

Other Organs

The muscles. The pathological changes in muscle are brought about in 2 ways (Hipps, 1942) Denervation gives rise to atrophy, degeneration, and replacement. Lesions can also occur secondarily from changing tension. Too much tension gives tears and resultant fibrosis, while too little gives changes identical with denervation These secondary lesions may produce just as much weakness in a muscle as the primary changes due to denervation. Secondary changes following immobility and disuse are more severe than those after overactivity. That primary changes may occur in the muscles, as believed by Kenny (see above) is partly suggested by the observations of Carey *et al.* (1944) on 3 cases dying of bulbar poliomyelitis They found practically complete denervation of the extrafusal muscle fibers at the myoneural junction in respiratory muscles, within 36 hours of the onset of symptoms This myoneural change was undoubtedly the terminal result of a pathological process occurring in the incubation period During the first week of illness, there was a striking passive hyperemia of the capillaries in some muscles Many fibers showed a granular and hyaline degeneration. There was a proliferation of the perivascular reticulo-endothelial mesenchyme leading to the differentiation of epithelial-like lipoblasts. The subcutaneous fat of the neck was undergoing regressive defatting changes and proliferation

The lymphatic system It has already been mentioned that there is considerable lymphatic hyperplasia in poliomyelitis Thus the Peyer's patches are ulcerated, the follicles prominent, and the spleen enlarged. Histologically, these structures are congested and the follicles are filled with granular necrotic material (see Burrows, 1920, 1931, International Committee, 1932).

The lungs The commonest cause of death is respiratory paralysis, and consequently there are found very frequently congestion and edema, or definite pneumonia (see International Committee, 1932, Saphir, 1945).

The remainder of the body. Some congestion and occasional cellular infiltrations are found in the heart, liver, and kidneys Saphir (1945) records the presence of myocarditis (see also Wenner and Paul, 1947)

Laboratory Diagnosis

The cerebrospinal fluid should be examined from the earliest stages of the infection, with particular reference to the cell count and estimation of protein

There is no object in endeavoring to isolate the virus from the cerebrospinal fluid If the case proves fatal, part of the cord should be retained for histological examination, and the remainder sent, in glycerol, to a laboratory where monkey inoculation can be performed Attempts may be made to isolate virus from the nasopharynx and stools, using the methods of concentration already described

In convalescence, the serum may be sent to a research laboratory for the virus neutralization test, and it may be possible to carry out a retrospective diagnosis in this way, if an acute phase sample is also available. It should be remembered, however, that many cases fail to develop a response, or that it may be delayed (Ch. LXXXVIII), and the test is of practically no value as a diagnostic measure.

CHAPTER LXXXIII

THE EPIDEMIOLOGY OF ACUTE ANTERIOR POLIOMYELITIS

GEOGRAPHICAL DISTRIBUTION

POLIOMYELITIS has an almost world-wide distribution as an endemic infection, and occurs in epidemic form chiefly in North America, Scandinavia, and Australia. During World War II, great interest was taken in serious outbreaks occurring in Malta, St. Helena, Mauritius, and S Africa. Another feature of interest was the much higher incidence of the disease in U. S. and British troops in India and the Middle East than at home stations It is being increasingly recognized that poliomyelitis occurs in tropical and subtropical countries as an endemic infection, although probably paralytic cases are less common in these warmer lands than in America, Scandinavia, and Australia. Certain general reviews on epidemiology may be consulted by those interested in this question (Dale, 1928[1], League of Nations, 1930, 1932, 1934, 1935, International Committee, 1932).

Particularly valuable information on epidemiology, which has been made much use of, was afforded by the *UNRRA Bulletin of Communicable Diseases*. The *Weekly Epidemiological Record* of the League of Nations and United Nations is also most valuable.

North America

Poliomyelitis has been endemic in Canada and the United States since the beginning of the century and presents a considerable problem Cases were seen in 1907, and it was thought that the infection was imported by European immigrants (Flexner, 1910, Fischer, 1917)

An average case rate in the United States is between 5 and 10 per 100,000 with [illegible] ie incidence [illegible] 1930–1945 [illegible] 1943, 1944, he states that in 1944 the rate was 14 per 100,000 (over 19,000 cases), a figure considerably above that of any preceding year, except 1916. In 1945 there were over 13,500 cases (Dauer, 1946) 1946 was also an epidemic year, and Dauer (1947) recorded 25,191 cases A feature of the disease in the United States has been the [illegible] less severely involved, since [illegible] canvass, found that 5.7 per [illegible] e, 3 0 per 1000 had residual paralysis

In Canada the annual incidence is usually around 3.0 per 100,000 In 1946 there were 2,500 cases

The following table gives some of the references to the main individual outbreaks of poliomyelitis in recent years In addition, the annual papers of Dauer give detailed information on the incidence of the disease in various states (1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, see also Davis, Weber, and Arey, 1941, Howitt, Buss and Shaffrath, 1942)

[1] References are appended at the conclusion of Ch LXXXVIII, p. 1026 et seq

TABLE 58

INCIDENCE OF POLIOMYELITIS IN RECENT YEARS IN NORTH AMERICA

1916	Philadelphia New York	Henry and Johnson (1934) Amoss, Taylor, and Witherbee (1919) Lavinder, Freeman, and Frost (1918)
1917	New York	Neal, Du Bois, and Abrahamson (1916–19)
1912–27	Generalized	Aycock (1929)
1928	Generalized	
1930–1	New York	Laidlaw (1932)
1932	Philadelphia	Lucchesi (1934), Stokes *et al.* (1935)
1933	Generalized	
1934	California	Kessel, Hoyt, and Fisk (1934), writers in a *Symposium on Poliomyelitis* (1934), Kessel *et al* (1936–7)
1935	California Massachusetts New York	Kessel *et al.* (1936–7) Legg (1937) Fischer and Stillerman (1937)
1936	Alabama Canada Chicago Kentucky	Wenner (1946) Donovan (1937) Harmon and Levine (1936–7) Lumsden (1936)
1936–7	Tennessee	Levy (1938)
1937	U S A Generalized (nearly 10,000 cases) Toronto and Ontario	Hampton (1938), *Bull. Off int Hyg publ* (1938) Hyland et al. (1938), Silverthorne, Hawke, and Brown (1941)
1938	Niagara	Stebbins, Gillick, and Ingraham (1939)
1938–9	Alberta	McGugan (1939)
1939	Detroit S Carolina	Top and Vaughan (1941) Weston (1940)
1939–40	Tennessee	Lumsden (1941)
1940	Washington	Powers (1941)
1941	Alabama Manitoba Mississippi Ohio	Casey (1942 *a*, *b*, 1945), Wenner (1946) Adamson and Dubo (1942), Donovan and Bowman (1942 *a*, *b*) Lumsden (1942) Kramer *et al* (1943)
1942	New York State Winnipeg	McClure and Langmuir (1942) McCormick (1942)
1943	Chicago Kansas Los Angeles Texas Utah	Sherman (1944) Steegman and Stephenson (1945) Swartout and Frank (1944) Bohl, and Irons (1944–5), Pearson *et al* (1945) Anderson (1946), Gebhardt and McKay (1946)

TABLE 38—*Continued*

INCIDENCE OF POLIOMYELITIS IN RECENT YEARS IN NORTH AMERICA

1944	Buffalo	Smith *et al* (1945)
	New York State and Pennsylvania	Landsberg (1945), Conway and Eigweed (1946), Pau (1947 *b*)
	N Carolina	Stevick (1946)
	Virginia	Roper (1946)
1945	Naval Training School	Goldstein, Hammon, and Viets (1946)
	New Jersey	Aisenberg (1946)
	Southern Army Post	Nicholson (1946)
	Utah	Anderson (1946), Anderson *et al* (1947)
1946	Minneapolis	Pohl (1947)

Central and South America

Outbreaks have been recorded in the last 15 years in *Argentine* (*Bol Of San Pan*, 1943, Bustamente, 1944, Maggi, 1945), *Brazil* (Parreiras, 1939–40), *Chile, Colombia, Costa Rica* (Montes de Oca, 1941, Nunez, 1945), *Cuba* (Beguez César, 1942), *Ecuador, El Salvador* (Allwood-Paredes, 1944), *Mexico* (Stuck and Loiselle, 1943), Nicaragua, Panama (Taylor, 1944), *Paraguay, Peru, Puerto Rico, Uruguay, Venezuela* (Arcila and Requena, 1938).

West Indies

The disease is endemic in Barbadoes, Jamaica, Tobago, and Trinidad

Great Britain and Eire

In Great Britain the disease is endemic and the average annual number of poliomyelitis and polioencephalitis cases is not more than about 1000 Outbreaks have occurred from time to time, as shown in Table 39

A number of small outbreaks has been recorded of recent years in Eire (Musgrave, 1942, Deeny and McCormack, 1946).

TABLE 39

MAIN OUTBREAKS OF POLIOMYELITIS IN GREAT BRITAIN

1897	Hertfordshire	MacNalty (1936)
1904	London	MacNalty
1911	Generalized	MacNalty
1913	North of England	MacNalty
1926	Broadstairs, Kent	Aycock (1927)
	Leicester, East and West of England	MacNalty
1928	Glasgow	Halliday (1930–1)
1930	Grimsby	MacNalty
1934	Berkshire, Somerset	MacNalty
1935	Gloucestershire	Editorial in *Medical Officer* (1936)
1936	Southall, London	Smithard (1937)
1938	Essex	*British Medical Journal* (1938), Barber (1938), Bullough (1938), Camps (1940)
	S Wales	Drury and Sladden (1939)
1940	Harrogate	Payne (1941), Miller and Wray (1941)
1945	In a camp	Conybeare (1946)

In addition readers may consult the following papers *Lancet* (1937), Kelleher (1940)

The disease was very prevalent in 1947, the highest incidence for many years (about 18 cases per 100,000)

Scandinavia and the Arctic Regions

Poliomyelitis first made its appearance as an epidemic disease in 1905 (Wickman, 1907 Harbitz and Scheel, 1907 *a*, *b*) Numerous cases occured in all parts of Scandinavia at this *time*, *and in* Sweden *in 1905* the case rate was 22 per 100,000. The disease was quiescent from 1907–1911, but flared up again in 1911–1913.

Articles discussing the epidemiology of poliomyelitis in Scandinavia are those of Wernstedt (*1924*); Nissen (*1935*, *1936*), Wennerberg (*1035*); Wulff (*1936*); and Gard (1937, 1938)

In *Denmark* there were epidemics in 1920, 1924–5, 1929, in 1933–4 the case rate in a very *severe outbreak* was 126 per 100,000 (Jensen, 1934–5), in 1937 (Henningsen, 1939, Rasch, 1939, Henningsen and Rasch, 1940); *1942* and 1944 were years of high incidence

In Finland epidemics occured in 1919, 1930, 1931, 1934, 1935, 1940, 1944, and 1945.

In *Norway* there were epidemics in 1929, 1934, in *1936* there was a severe epidemic (Holst, 1938), in 1941, 1944, and 1945.

In *Sweden* epidemics occurred in 1923, 1924, 1928, 1929, 1930, 1932, 1934, 1935, 1936 (Kling, 1937, 1938 *a*), 1937–8 (*Bull. Off. int. Hyg. publ*, 1938, *30*, 2446), in 1943–1944 (*Dhunér*, *1944*), in 1945 Olin and Hemertz (1943) discuss the incidence from 1930–1939

Cases have been reported in *Greenland* (Hrolv, 1935, Høygaard, 1939); and *Iceland*, *especially in 1945*.

Europe

In France there was a considerable outbreak in the Bas-Rhin in 1930 (Levaditi, Schmutz, and Willemin, 1930), outbreaks occurred in 1943 and 1946 (see *J Amer. med. Ass*, 1947)

In *Germany*, from 1918 to 1924, cases occurred sporadically, but since that date there have been numerous epidemics, for instance, in 1932 there were approximately 4,000, and in 1934, 1,600 cases (see Mollers, 1936–7, Pohlen 1934, 1936 *b*; *British Medical Journal*, 1935). The disease was prevalent from 1936 to 1938 (Meier, 1938, *Reichsgesundheitsblatt*, 1938, Fey, 1939), epidemics occurred in 1939, 1941, and 1942 Austria has experienced various outbreaks (see Mayer, 1934, Khaum, 1937, Siegel, 1937, Zappert, 1937).

Outbreaks have occurred in the last 15 years in *Belgium*, especially in 1939 and 1945 (Lancet, *1945*), *Holland*, especially in 1943 and 1944, *Spain* (Albaladejo, 1936), *Italy* (Petragnani, 1937), *Roumania* (Marinesco, Manicatide, and Draganesco, *1929*), *Bulgaria*, *Hungary*, *Czechoslovakia* (Lancet, 1945), *Yugoslavia* (Djourichitch, 1937), Poland, U.S.S.R.

In *Switzerland*, the disease is becoming increasingly prevalent (*Bull Serv Féd. Hyg publ.*, 1936, Alder, 1937, Wieland, 1937, 1938, Exchaquet, 1938, Stahel, 1938, Abegg, 1942, Fanconi and Zellweger, 1942, Zellweger, 1945). Recent epidemics were those of 1941, 1942, and 1944

The Mediterranean Area

The disease is endemic on the *N African seaboard*, in Algeria, Morocco, and Tunis *In Egypt* the disease is endemic, and affects chiefly children under 5; adult natives are rarely attacked (Paul, Havens, and van Rooyen, *1944*)

In World War II, there was a high incidence of poliomyelitis of severe type in the *U.S*, *British, and Dominion Expeditionary Forces*, the incidence was about *10* times that in home commands (van Rooyen and Morgan, 1943, Paul, Havens, and van Rooyen, 1944, Illingworth, 1945, McAlpine, 1945, Caughey and Porteous, 1946, van Rooyen and Kirk, 1946)

In *Palestine* it is probable that the disease has been endemic since Biblical times The highest incidence is said to be in Jews, chiefly infants, Arabs are rarely attacked (Levy, 1937, Paul, Havens, and van Rooyen, 1944, Gruenfelder, *1946*)

Malta and Gozo. Poliomyelitis is endemic in Malta, and from *1921–1941* there were about 60 cases A severe epidemic began on November 15th, 1942 in Malta, on November 21st in Gozo, on the 27th Service personnel were attacked The epidemic ended *in* June, 1943 (Agius *et al*, 1945, Bernard, 1945, Bernstein, Clark, and Tunbridge, 1945, Debono, 1945, Seddon *et al*, 1945, Morana, 1945) Eighty per cent of the cases were aged under 5, and only 6 cases were over 10, the highest incidence was at 2 and 3 years, but many infants were attacked There were 426 civilian cases, and 57 in the Services Of 61 adults only 4 were Maltese The civilian fatality rate was 3 7 per cent and the service 19 3 per cent.

The etiology of the outbreak was obscure, but possible factors were overcrowding, physical fatigue, malnutrition resulting from the effects of aerial bombardment on an

unprecedented scale use of sewage to manure crops (Seddon *et al*; Morana). It was not possible to assess the relative part played by droplets or feces in the dissemination of the infection, and perhaps both channels were involved. Discussing the outbreak, van R[illegible] and Kirk (1946) concluded that fecal spread was the more [illegible] the strain of virus may have been [illegible] by service personnel. Neverthel[illegible] adult Maltese were immune.

Malta had a smaller outbre[illegible]ning in November, 1945, again affecting chiefly children under 5 (Kauntze, 1946).

Africa

Poliomyelitis appears to be endemic in the Belgian Congo (Vilen, 1942) and French Equatorial Africa (Ceccaldi, 1943). The disease occurs in West Africa, and affects chiefly children (Olitsky and Findlay, 1946), cases have occurred in Europeans, especially troops (Findlay, Anderson, and Haggie, 1946). The disease is common in the rainy season, as are amebiasis and hepatitis.

The disease has been described in N. Rhodesia (Gear and Rodger, 1946), and S. Rhodesia (Ratchken, 1944).

Of recent years, poliomyelitis has become more prevalent in the Union of South Africa, and numerous outbreaks have occurred (Gear, Yeo, and Mundel, 1945, Henson and Post, 1945, Kaplan, 1945, Turner, 1945, Gear, 1946, Gear and Mundel, 1946, Gear, Mundel, and Wilson, 1946, and the Report of the Union Department of Public Health, 1945).

Poliomyelitis was first noted in South Africa in 1917–18, when there was a severe outbreak. The incidence of the disease remained low until February, 1944, when there was an outbreak in Johannesburg. In September, 1944, Durban became involved, and the disease spread to other parts. The maximum incidence of the disease was in November and December, and for the year ended June 30, 1945 there were over 1,300 cases in the Union. It is possible that movements occasioned by the war introduced a virulent strain from the Middle East, and favored its spread.

St Helena

St Helena was visited by a severe outbreak during World War II (*Colonial Office Report*, 1945–6, Kauntze, 1946). There were 216 cases, 215 in Islanders and 1 in a European, between November 14th, 1945 and January 26th, 1946. Abortive and paralytic cases occurred in the proportion of 7:3. There were few cases under 5, and the majority was aged 10–25. Most deaths ocurred in the 15–20 age group. It appeared, therefore, that the virus was a "foreign" strain, perhaps introduced from South Africa, invading a population not immunized by previous exposure. A visit from a troopship appeared to be responsible. The state of nutrition was low at the time, owing to drought.

Mauritius

The disease is endemic in this island. In the first 5 months of 1945 there was a severe outbreak of 1,018 cases, the attack rate was 2.4 per 1,000 (McFarlan, 1946, McFarlan, Dick, and Seddon, 1946, Seddon, Hawes, and Raffray, 1946). Practically all the cases were under 10 and chiefly under 5. The evidence suggested spread of infection largely by healthy adults infected, probably in the nasopharynx, for a short time only. The age distribution suggested an epidemic "flare-up" of an endemic infection. There had been severe cyclone damage shortly before the outbreak.

India, the Pacific, and the East

The disease is endemic in India and affects chiefly children, adults being rarely involved, epidemics are unusual. During the second world war, from 1942–44, European and American troops had a considerably higher incidence of attack than in home bases, the disease was rare in British troops from 1938–41, and in Indian troops at all times (McAlpine, 1945–1946).

Sporadic cases occur in China (New, 1934, Scott, 1938, Yen and Hsu, 1941), Indo-China (Canet, 1936), Hawaii (Lee, 1941, Doolittle, 1943). Paul (1947 *a*) reports that despite the relative absence of epidemics in Japan, the average annual mortality rate is similar to that of the United States.

There was an outbreak in Malaya (in Singapore) in 1945–6, with approximately 85 cases, 60 being children under the age of 5, and 22 being Service personnel (Kauntze, 1946).

Australasia

Epidemics occurred in Australia in 1929, 1931, 1932, 1934. In 1937–8 over 2,000 cases occurred in Melbourne and Victoria (*Circular on Infantile Paralysis*, 1937, *Health*, 1937, 1938; *Health Bulletin*, 1937, 1938, Bull, 1937–8, *Med. J. Aust*, 1938; Burnet, 1940; Williams, 1940); Sydney was also involved (Hamilton, 1940), and New South Wales (Helms, 1941 *a*). There was an outbreak in 1945–6

New Zealand had an outbreak in 1936–7 (James, 1937; Watt, 1938).

AGE, SEX, AND RACIAL INCIDENCE

Age

Poliomyelitis outbreaks tend to show one of three common types of age incidence.

1. *With main attack on the preschool child.* Many poliomyelitis outbreaks have affected predominantly those under 5, hence the term "infantile" paralysis This type of incidence was usual in the cities and towns of the United States until 1918. Thus in the 1916 New York epidemic, and in Philadelphia, about 90 per cent of cases were under 5. More recently in the United States the incidence has changed (see below).

In the 1942–3 Malta outbreak, about 90 per cent. of cases were under 5 In the 1945 Mauritius epidemic the attack rate under 1 year was 4 per 1,000, at 1–5 it was 12 per 1,000, and then fell rapidly. In S Africa in 1944–5 the greatest incidence was in the 1–4 age group. These outbreaks, all, it should be noted, in warm climates, [illegible] an endemic infection This typc [illegible] such an occurrence. The sam [illegible] t and Japan in non-epidemic periods (see, e g., Paul, 1947 *a*). The virus is probably widespread in such *communities, and gives rise to immunity largely by subclinical* attack. Such a virus is presumably of high infectivity but of low paralysis-producing power (see Burnet, 1940) By the age of about 5, most of [illegible] the only susceptibles are the younges [illegible] incidence of any infection in infancy [illegible] of virus in the environment, which means that adults and older children are excreting it.

It is possible, however, that in poliomyelitis the acquisition of resistance with age is due to the development of physiological barrier mechanisms independent altogether of active immunity

It has been suggested that on first introduction to an area, poliomyelitis may also tend to give this type of age distribution Thus, in the 1943 El Salvador (S. America) outbreak, 90 per cent. of patients were under 5 (Allwood-Paredes, 1944).

Very young infants are not immune, and cases have been reported in newborn babies (Biermann and Piszczek, 1944, Frovig, 1947), and in one 4 *months old* (Hess, Levinson, and Hess, 1942)

2 *With main attack on the older child* A remarkable feature of urban poliomyelitis in the last 30 years in America, Australia, and Scandinavia has been the increasing tendency to involve the school as opposed to the preschool child In this way the age incidence resembles that of measles and diphtheria (Aycock, 1928, Burnet, 1940). Thus, in 1931 in New York, Laidlaw (1932) found twice as many cases over 5 as there were in 1916 In Philadelphia in 1932, 43 per cent were over 5, whereas in 1916 only 12 per cent. were over this age (Henry and Johnson, 1934). In Denmark in 1944, 53 per cent were over 15 (Horstmann, 1946), in 1934 there were 31 per cent over this age, and in 1911–14 only 10 per cent were over 15 (Jensen, 1934–5) The same increase in the percentage of older children attacked has been noted in Australia (see, e g, Burnet, 1940, Helms, 1941 *a*), and Switzer-

land (Fanconi and Zellweger, 1942; Zellweger, 1945). The phenomenon cannot be explained by the falling birth rate (Stocks, 1936). Other authors have confirmed this increasing tendency to affect the older child (Nissen, 1936, Dauer, 1938, Payne, 1941, Donovan and Bowman, 1942 *b*, Sherman, 1944; Landsberg, 1945; Conway and Bigwood, 1946). Burnet (1940) points out that the age incidence of urban poliomyelitis now resembles that of rural poliomyelitis in an earlier period

As regards America, the position is not quite as simple as has been stated, for differences exist between the North and South For example, investigating the disease in Alabama, in the Southern States, Wenner (1946) found that children under 5 were 3 times as commonly attacked as those aged 5–10, whereas in Connecticut at the same period, the 5–10 group was attacked at least as frequently as those under 5.

In the 1945–6 St. Helena outbreak there were few cases under 5, the majority being aged 6–20. It was suggested, therefore, that the causal virus was introduced from outside the Colony, and invaded a susceptible population not immunized by previous exposure, the "under fives" being presumably less exposed to infection

It has been suggested that the explanation of this increasing attack on older children is largely improved hygiene, the young child now having less opportunity of acquiring infection (Fanconi and Zellweger, 1942). Burnet (1945 *b*) points out that the changed age incidence has been largely apparent in the more highly civilized countries where improved social technique has diminished the spread of intestinal and respiratory infections in general. He holds that there are no grounds for believing that children under 3 are not still just as susceptible to the paralytic type of infection as older children, the difference from 30 years ago must be due to their less frequent infection

We may provisionally state, therefore, that a high attack rate on the under fives is characteristic of areas with poor social hygiene where there are frequent opportunities for infection of these children It should be noted that recent outbreaks with main incidence on the under fives have been in warm climates A high attack rate on the school child is characteristic of areas with a better social hygiene, where infection occurs later in life.

3. *With main attack on adults* Adults are not commonly attacked, and it is presumed that this is due to resistance acquired in childhood For example, in the 1944 N Carolina outbreak only 6 per cent. of cases were over 20 (Stevick, 1946). A number of outbreaks has, however, been recorded where about a third of the cases has been over 15 years of age; in certain outbreaks in reputedly "virgin soil" the main incidence has been on young adults (International Committee, 1932; Høygaard, 1939).

During World War II, European troops in the Middle East and India showed a much higher incidence than in home bases, and the mortality rate was high. Presumably these men were exposed to a virus to which they had no immunity. An interesting feature was the approximately 5 times greater incidence in officers, this was noted in India and the various Mediterranean zones (McAlpine, 1945; van Rooyen and Kirk, 1946) A possible explanation was the often poorer standard of hygiene in officers' messes, further there was a greater tendency for these ranks to feed at hotels and cafés

Other outbreaks have involved service men in the U. S. A (Goldstein, Hammon, and Viets, 1946, Nicholson, 1946)

There is probably no upper limit of susceptibility, and cases have been reported in persons aged 68 (Fox, 1938), and 69 (Lee, 1941).

Sex

The disease has usually shown an increased incidence in males as compared to females The International Committee (1932) summarized the published data relative to some 36,000 cases and found the male female ratio was 1.3 1 (see also

Henningsen and Rasch, 1940; Donovan and Bowman, 1942 *b*, Collins, 1946). While this ratio holds for younger children, it has been stated that after 20 years of age there are more cases in females than males

Race

Although poliomyelitis probably occurs in all parts of the world, certain races seem to be less susceptible to paralytic infection than white people. Thus in the United States the reported incidence in white persons is 3–4 times that in colored (International Committee, 1932, Harmon, 1936; Taylor, 1944, Collins, 1946, Stevick, 1946). In S. Africa, Gear (1946) found Europeans to be 10 times more susceptible than Africans

In Hawaii a higher incidence has been reported in Caucasian and Hawaiian groups than in Filipinos, Chinese, and Japanese (Lee, 1941, Doolittle, 1943).

The varying susceptibility of different races may sometimes, at any rate, be partly explicable by the nature of their work, or social status. Thus in the 1945 Mauritius outbreak, the Chinese showed a higher incidence, but probably because being largely shopkeepers they had more frequent contacts outside their families (McFarlan, 1946, McFarlan, Dick, and Seddon, 1946).

In other cases an apparent varying susceptibility in different races may be explained in terms of immunity. Thus in the Malta outbreak, cases occurred in British service personnel, but not in Maltese troops serving alongside Elsewhere in the Middle East, cases occurred in troops but not commonly in adult natives. The same situation was observed in India In these areas latent immunization protected the adults against the local strains of virus

It would seem then, that differences in racial susceptibility may be partly inherent, partly dependent on opportunities of contracting infection, and partly dependent on previous immunization

RELATIVE DISTRIBUTION OF CASES IN URBAN AND RURAL AREAS

Many observers have referred to a higher incidence in country districts than in towns and cities (Wickman, 1907, Lavinder, Freeman, and Frost, 1918, Levaditi, Schmutz, and Willemin, 1930, *International Committee*, 1932, Nissen, 1935, Canet, 1936, Lumsden, 1936, Root, 1936, Wulff, 1936, Petersen, 1941, Olin and Heinertz, 1943, Paul, 1947 *b*) It has been suggested that this is because resistance follows immunization by latent infection, which infection is naturally more prevalent in areas with the denser populations, than in rural areas where human contacts are less. Further, there is a tendency for a different age incidence in rural areas as compared to towns In rural areas, where immunization is less, one might expect the disease to attack persons of a somewhat older age than in towns, where the main susceptibles would be young people Gard (1938), in Sweden, found that in country districts most of the cases were aged 15–30, while in towns they were chiefly under 5 Paul (1947 *b*), investigating an outbreak in New York State and Pennsylvania in 1944, found that in the rural areas the older age groups had a higher incidence than was the case in urban districts This feature was not, however, noted in the 1937–8 Victorian epidemic (Burnet, 1940) In the 1945 Mauritius outbreak, there was a lower incidence in town dwellers at all ages, which suggested that the immunity in these persons was acquired earlier in life

SEASONAL DISTRIBUTION

The tendency for cases to occur predominantly in the summer and autumn in the northern hemisphere, and in the first 4 months of the year in the southern hemisphere has been recognized for a considerable time In the United States the median date of the peak week of notifications centers round August 15th in the South and September 12th in the North and West (Collins, 1946, see also Aycock,

1929) Quite a number of outbreaks has been reported as continuing into the colder months, and the report of the International Committee (1932) mentions approximately 40 such outbreaks. The 1933–4 Danish epidemic, for instance, showed a number of cases occurring in the early winter, and such was the experience of Wennerberg in Gothenburg (1935), the 1938 British epidemic also lasted into the winter (*Brit. med. J*, 1938), epidemics arising *de novo* in the winter are very unusual (see International Committee, 1932, McGugan, 1939) Dauer (1941) reports that when there is an increased prevalence in the late autumn or early winter, especially in a localized area, a more widespread epidemic is likely to follow in the summer.

Gard (1938) has stressed the rôle of certain meteorological factors in Swedish outbreaks. Thus, he found that an epidemic ceased when the temperature fell to 0 C., only to flare up again in March when the reading began to rise He also found that poliomyelitis epidemics consisted of small waves, and that the peak in each wave was preceded by an increase of rainfall, usually 14 to 20 days before (1937, 1938) Further information on the possible effect of meteorological conditions on the incidence of poliomyelitis would be of interest. For example, the study of saturation deficiency might throw some light on the importance of droplet infection

Neegaard (1942) has noted a rapid increase of cases in periods of weather apparently favorable. It may be that susceptibility is in some way increased in the autumn months (see Muller, 1945)

THE ECOLOGY OF POLIOMYELITIS

Of recent years it has become possible to visualize the essential biological character of poliomyelitis as an ecological problem. Thus Muller (1941) postulates a widespread distribution of the virus in epidemic periods Many persons are infected, and the virus reaches the CNS In some cases no significant symptoms result, while in others there are signs of abortive poliomyelitis In others, owing to some impairment of the relationship between the virus and the host, paralysis develops

Gard (1943 *b*), however, suggests that the course of infection resembles that in Theiler's disease, in that the virus multiplies in the intestine, and only exceptionally invades the nervous system

Paralysis may be regarded as an unfortunate complication from the point of view of the host, but of no ecological significance to the parasite (Burnet, 1945 *b*, Kendall, 1945).

Burnet (1945 *a*, *b*) has given much thought to this matter, and has formulated the following conception The virus is essentially a low-grade parasite of the pharynx, tonsils, and small intestine of children In nonepidemic times it is usually passed by fecal contamination and causes subclinical infection The virus survives as a species because it can be readily implanted in the pharynx and intestine of children, is excreted in feces, and is relatively resistant to destructive influences. Epidemic strains are endowed with the property of more readily causing paralysis, and have a greater potentiality for spread by the respiratory tract These strains may be regarded as biologically irrelevant mutations to a more invasive type However, the term "invasive" is relative, and the incidence of paralysis depends on unknown factors in the host

Burnet suggests that the virus has developed new biological potentialities in the 20th century Perhaps the virus evolved primarily as a parasite of the intestine of small rodents A chance mutant may have found its way to a human being by food contaminated with rodent feces, and produced disease At first, perhaps, the disease spread only from rodent to man, but eventually a variant arose which spread from man to man by the intestine or pharynx.

It is possible that a strain of low invasiveness growing in the epithelium of the gut would "blockade" the cells against a more invasive strain (Evans and Green, 1947)

ORIGIN OF EPIDEMICS AND OUTBREAKS

Poliomyelitis is endemic in many parts of the world, perhaps in almost all. From time to time epidemics occur, for reasons that are quite obscure. It is commonly believed that in areas where the disease is endemic most persons acquire immunity, chiefly by subclinical infection, fairly early in life The chief incidence is, therefore, on the under fives, and it may be postulated that epidemics occur when sufficient numbers of susceptibles have accumulated In certain parts of the world, owing to a higher standard of social hygiene, the attack is postponed to a later age

Epidemics may be precipitated by various causes. Thus in Malta there had been a long period of privation and hardship resulting from aerial bombardment, an additional factor was the recent introduction of human manure as a fertilizer. In Mauritius, cyclone damage occurred prior to the outbreak, and increased the overcrowding, a high incidence of intestinal infections was noted before the outbreak. Climate seems to play an important part. The rôle of such factors is probably to facilitate transfer of the causal agent and thus increase its virulence by passage.

According to this view, the causal agent of an outbreak is essentially the same as the endemic strain in antigenic structure, very likely some variant has developed which is endowed with properties that make it readily transmissible and of enhanced virulence (Burnet, 1945 *b*) "Foreign" adults entering such endemic areas may be susceptible to infection This point was demonstrated in the World War II, where the incidence of poliomyelitis in U. S. and British troops in the Middle East and India was about 10 times that in home commands.

Rather than a single strain being responsible, Aycock (1942 *c*) suggests that several may be in circulation and attain wide distribution at one time. A given outbreak may be precipitated by various factors, on a background of carriage of several strains. The absence of any simple method of "typing" strains of virus makes any such speculation difficult of general proof.

An alternative explanation for the origin of an epidemic is that the strain of virus may be imported Thus it was believed that the 1937–8 Australian epidemic was due to a strain from New Zealand The 1945–6 St. Helena outbreak was thought to have been caused by a strain introduced from S Africa, while the disease in this latter country in 1944–5 was held to be due to a strain imported from the Middle East or the U S A An outbreak in N Rhodesia was also attributed to introduction of a foreign strain In fact, the Middle East played a prominent part in the epidemiology of poliomyelitis in the World War II

Poliomyelitis has a pronounced seasonal incidence, and one has to consider how the infection is maintained in between outbreaks There is no evidence that virus is excreted by any considerable number of the community for long after the end of an epidemic (Wenner and Casey, 1943), and sewage in nonepidemic periods is usually virus-free It is probable that sporadic, chiefly abortive cases, occur throughout the year with sufficient frequency for the virus to be kept alive by passage in human beings There is no evidence, as yet, of an extrahuman reservoir of infection, for example, in a lower animal or arthropod It should be remembered, however, that the virus can probably survive outside the body in suitable conditions, for example, in water, for some months

In Buffalo in 1944, Smith *et al* (1945) inquired into the circumstances surrounding the development of the first 3 paralytic cases of the seasonal outbreak There was no evidence that these cases arose *de novo*, they were relatively late developments in a cycle in progress for about 3 months There was only an interval of 2 months between the last reported case in 1943, and the first probable case of abortive poliomyelitis in 1944 Owing to the difficulty in diagnosing abortive polio-

myelitis, they thought that this type of illness was probably occurring all the time between the 2 outbreaks.

Paul (1947 *b*) investigated an epidemic which evidently had its origin in a few paralytic cases in 2 townships in the winter of 1943–44 (October to May), 30 more cases appeared in the surrounding area in June-August, 1944; from June onward the epidemic quickly radiated outward.

Confirmation of the point that the infection may be present in a locality some weeks before the occurrence of the first case has been given by Gear and Rodger (1946), who in N Rhodesia found that persons visiting the locality in this period developed symptoms on return home.

Levaditi (1946) visualizes a widespread dispersal of virus at the onset of an epidemic, associated with an increasing neurotropism, and a changed susceptibility in the population The immune persons who become infected act as healthy carriers, the fully susceptible develop paralysis, the partly susceptible develop abortive infection

SOURCES AND MODES OF INFECTION

Infection with the virus of poliomyelitis can be contracted in many ways In some cases, the infection is autogenous, in others it is evidently contracted from food or milk; in many others, contact with a previous sufferer or close contact is responsible. In some instances healthy carriers are responsible. The rôle of arthropods, especially flies, has also been investigated, and must be considered seriously. The question arises in almost all cases whether the virus is transferred by the medium of feces or nasopharyngeal droplets.

Before discussing the various alternative methods of spread, it may be helpful to recapitulate very briefly what is known concerning the excretion of poliomyelitis virus from the human body.

Excretion of Poliomyelitis Virus by Cases and Contacts

This question is fully discussed on page 928 et seq., where it is shown that the essential points of importance in epidemiology are as follows

1 Virus is present in the nasopharynx of the case, whether abortive or paralytic, until about 3 days after onset A longer period of infectivity is unusual

2. Virus is present in the feces of the case, abortive or paralytic, for about 1–2 months

3 True convalescent carriers are very rare

4 Virus is widely distributed in the environment of abortive or paralytic cases It has been recovered most commonly from intimate contacts, such as members of the same family or playmates, and more usually from children than adults In these contacts, the stool remains infected longer than the nasopharynx

5. To summarize, the virus is known to be present in the nasopharynx, and for a longer time in the feces, of abortive and paralytic cases, and of intimate contacts—mainly children but including adults

Method of Transfer of Infection in Poliomyelitis

We have already pointed out that a study of the portal of entry of the virus may throw light on the method of transfer The result of modern histological observations is to emphasize the rarity of entry through the olfactory area and stress the importance of entry through the pharynx and small intestine The pharynx can be infected either by inhaled material passing downward, or by ingested material It is questionable, therefore, whether this work helps very much in deciding how the virus enters the body of the patient It is clear, however, that inhalation is not as common as ingestion.

Infected persons can give rise to infection in others by means of droplets of nasopharyngeal secretion or by feces.

Until a few years ago it was generally held that poliomyelitis was transmitted by droplets. The fact that the virus was present in the nasopharynx, and that nasopharyngeal symptoms were common in the prodromal phase were powerful arguments, at that time it was not realized that virus was also present in the feces. In certain recent outbreaks, experienced workers, after weighing the evidence, have concluded that droplet infection was the most likely explanation. This opinion was reached by those studying the 1937–8 Australia, the 1942–3 Malta, and the 1945 Mauritius outbreaks.

There have always been difficulties in accepting the droplet theory. For example, the seasonal incidence is unlike that of other droplet infections. Institutional and school outbreaks have always been unusual—a marked contrast to the state of affairs with influenza or measles.

In the last 10 years or so, evidence has increased that poliomyelitis is more usually spread by fecal material. In some cases, the fecal material may be ingested with food contaminated by flies, or by the washing of fruit and vegetables in sewage-polluted water, water supplies may be infected. This type of spread probably operates commonly in the subtropics and tropics, although it is unlikely that public water supplies are often concerned. Perhaps more usually the fecal matter is transferred by actual contact with the hands of the excreter, or by fomites such as soap and towels. It has been shown that the virus is usually spread to close familial contacts and playmates. Fairly prolonged and direct contact is necessary for the transmission of infection. The position would seem to be analogous to the transfer of dysentery bacilli, e.g., Sonne's bacillus. The seasonal incidence is certainly more in favor of an intestinal than a respiratory infection. It has been held that the rapidity of spread in certain outbreaks, e.g., in Malta in 1942–3, is against the fecal method of transfer, but the behavior of Sonne dysentery in recent years has been somewhat similar.

Burnet (1945 *b*) has suggested that in nonepidemic periods the virus is transmitted chiefly by fecal contamination. In epidemic periods, however, it is more usually spread by droplets.

Autogenous Infection

In a small percentage of cases, infection is precipitated by various factors (see page 919, of which tonsillectomy is the best ... esumed that the ... invades it shortly ... stools of norma ... likely that autogenous infection operates at all commonly.

Gard (1943 *a*), who has isolated a noninfective "intestinal protein" from the feces of healthy men and mice, suggests that this is a virus normally inhabiting the gut. It may be an invader of intestinal micro-organisms rather than the human or mouse body cells. Virus is continually absorbed and deposited in the lymph glands, where it is gradually destroyed. Th ... is is not neurotropic and normally ... strated to be pathogenic on ex ... under certain conditions, neurotropic variants develop. In mice, this may occur at weaning, in man climatic factors may be responsible. According to this view, poliomyelitis is in a sense an autogenous infection, although exogenous factors may precipitate an attack.

The Acute Case

The case of poliomyelitis, abortive or paralytic, excretes the virus in the stool for some weeks, and virus is also present in the nasopharynx for some days. Evidently, therefore, such persons may infect others. Careful epidemiological inquiries have shown that the patient is probably infectious for 4–5 days before, to 4–5 days after, the onset of symptoms (Aycock and Kessel, 1943). Casey (1942 *a*, *b*) found

the period to be 3 days before and after. The nonparalytic case is particularly dangerous, as he is probably not confined to bed The potential infectivity of the acute case has for long been recognized, but in the past it was not the general experience that these persons were responsible for spreading the infection to any considerable extent. For example, the International Committee (1932) stated that the lack of obvious connection between cases was "one of the striking and constant features of the epidemiology." Even 20–30 years ago, however, contact between cases was demonstrated in a number of outbreaks (see International Committee).

Of recent years the evidence has become increasingly strong that the source of infection frequently can be traced to fairly intimate contact with a previous case, abortive or paralytic, in the infectious period. It is generally believed that the young child, under 5, is more infective than the older child

Epidemiological inquiries have shown that a case has often played with a child in the infectious period Numerous workers have supported this concept of transmission of infection by close personal association with sufferers (Nicoll, 1917, Bingold, 1936, Langmuir, 1942, Burnet, 1940, 1945 *b*, Anderson, 1946; Gear and Rodger, 1946).

In the 1937–8 Victoria epidemic infection was thought to arise in this way in the bulk of cases. The hospital outpatient department was believed to be a common disseminating center. It was concluded that child contact was the main method of spread, and that adults were seldom involved (Bull, 1937–8)

Important observations were made in a rural outbreak in Alabama in 1941 by Casey (1942 *a*, *b*) He found that during the 3 days before the onset of symptoms, 81/101 cases had prolonged direct contact with a patient suffering from an illness compatible with poliomyelitis He found that human travel was the essential factor. The transmitters of the infection were almost all children under 15, mostly under 4 When the average distance (by air) of the places of contact for each 12 day period of the epidemic was calculated, there was evidence of a radial spread of infection at the rate of about 1 mile per 10–11 days (Casey, 1945)

In an epidemic in New York State and Pennsylvania in 1944, Paul (1947 *b*) showed that the spread of infection was outward from a central focus, traveling approximately 0.5 mile a day

From the days of Wickman, it has been known that poliomyelitis tends to spread along routes of travel, the spread being essentially determined by human intercourse

Langmuir (1942) made a survey of each home within 2 miles radius of 3 affected families The stools of 4/8 adults, and 16/19 children who were intimate contacts were found to be positive

An interesting investigation was made in urban Chicago by Casey, Fishbein, and Bundesen (1945). They made a census of all persons under 25 in an area within one block of a case, and made a comparison with a control area in a distant part of the city Of 66 persons in contact with poliomyelitis cases during the infectious period, 37 developed illnesses 6–15 days later that were compatible with a diagnosis of poliomyelitis, 24 persons were definitely diagnosed Later Casey *et al.* (1947) studied 22 contacts of cases, and found that 14 developed fever, presumably due to poliomyelitis, within 5–25 days of exposure

Pearson *et al* (1945) investigated the distribution of poliomyelitis virus in stools at Fort Worth They found virus in familial contacts very commonly, much less commonly in nonfamilial contacts, and only very rarely in noncontacts

In the Mauritius 1945 epidemic, 50 per cent of cases had direct or indirect contact with paralytic cases or with areas where the disease was occurring (McFarlan, 1940, McFarlan, Dick, and Seddon, 1946)

In outbreaks in the State of Utah in 1943 and 1945, Anderson (1946) found direct contact quite frequently

Further evidence supporting the spread of infection by close personal contact

with a case, is afforded by a study of familial and institutional outbreaks A very special instance is the contraction of poliomyelitis by nurses and laboratory workers (see below).

Familial infections.

Until recently, it was unusual to find more than 1 case per family. For example, in the 1916 New York epidemic, 44 children were exposed to infection in their homes, but only 6 3 per cent. developed symptoms (Neal, Du Bois, and Abrahamson, 1916–9). In Roumania it was found that only in 5 families out of 50 investigated could home contact be said to have given rise to a second case (Marinesco, Manicatide, and Draganesco, 1939). Investigating 400 cases in the Department of Bas-Rhin (Alsace) in 1930, Levaditi, Schmutz, and Willemin found no case of family infection.

More recently, contact in the home seems to have played a more important part, and numerous authors have recorded instances of 2, and much less commonly 3 ... Camps, 1940, Strangmann, 1940, Ay- c ... Gear and Mundel, 1946; Gear and I ... 1937–8 epidemic, 24 per cent. of the ε ... 1937–8). In some instances, doubtless, the 2 cases have been infected from the same source (Gard, 1938); in others it appears that the second case has been infected by the first.

Familial infections, as would be expected, are commoner when the exposed children are young than when they are over 10.

An interesting study of over 200 families in a town in which poliomyelitis was occurring was made by Paul, Salinger, and Trask (1932). In each of these families there had been at least one case of frank poliomyelitis The other susceptible children in the homes were found to show a high percentage of minor illnesses; thus, 39 per cent. of children from 1 to 4 years old and 32 per cent. from 5 to 9 had such attacks. Only 9 per cent. of children in homes where there had been no case of poliomyelitis showed similar illnesses. The evidence was most suggestive that the contacts were, in fact, suffering from nonparalytic attacks of poliomyelitis Somewhat similar studies were reported by Kramer and Aycock (1931–2).

A remarkable example of the wide distribution of virus that may occur in families and their contacts was afforded by the experience of Francis *et al.* (1942). Five children of one family developed bulbar paralysis after tonsillectomy. A 6th child, who was not operated upon, remained healthy, but excreted virus in the stool The virus was recovered from the stools of 2 cousins with whom 2 of the children had lived a month before An additional 4 cousins of another group with whom the children had lived later also carried virus, but showed no symptoms No virus was recovered from adult relatives, 3/28 playmates excreted the virus

Although there has been this increased tendency to familial cases in certain ... Malta in 1942–3 ... 945 ... mag- ... atly increased (Swartout and Frank, 1944).

Institutional outbreaks.

Although cases of poliomyelitis have frequently been nursed in general wards without any particular "barrier" precautions, institutional outbreaks have been rare. Outbreaks in various types of institution have, however, been recorded from time to time. Thus, numerous authors have described outbreaks in schools (Wickman, 1907; Halliday, 1930–1, Siegl, 1937, de Rudder and Peterson, 1938, Somerville, 1938, Wolter, 1938) In Great Britain, such outbreaks have been unusual and have seldom

involved more than a few boys, secondary cases have been few in number (*res. Counc. spec. Rep*, *No.* 227; Barber, 1938, Smith, 1939).

Outbreaks have also occurred in *children's homes* of various types (International Committee, 1932, Kramer, Gilliam, and Molner, 1939, Silverman, 1941; Gear, Yeo, and Mundel, 1945)

Camps and holiday homes have also been involved (Lagrange, 1939, Brown, Francis, and Pearson, 1945, Conybeare, 1946, Melnick, Horstmann, and Ward, 1946).

The evidence is that in these institutional outbreaks a large number of children and a lesser number of adult contacts become infected, and excrete the virus in the stool, a few develop abortive poliomyelitis, and still fewer paralytic symptoms. A few examples may be quoted

Virus was recovered from the stools of 5/6 boy contacts at a camp outbreak who must all have acquired their infection within 6 days of exposure, virus was recovered from the throats of 2/3 boys with positive stools (Brown, Francis, and Pearson, 1945)

In an outbreak of 5 cases in a home the virus was recovered from the stools of 3/12 healthy children, and from 2/3 who had a probable abortive attack; in all, 10/20 children harbored the virus at some time during a 30 day period (Kramer, Gilliam, and Molner, 1939) In this outbreak one nurse in close contact had virus in the stool

In a children's home where one case occurred, 2/7 healthy contacts excreted the virus in the feces (Gear, Yeo, and Mundel, 1945). In a boys' camp of 75 persons there were 4 cases of paralytic infection The virus was recovered from 1 pool of oropharyngeal washings, and 3 pools of stools. Some of the infective material derived from boys who had symptoms suggestive of an abortive attack, and some from boys who had no symptoms (Melnick, Horstmann, and Ward, 1946).

The number of cases of infection found in contacts depends partly on the thoroughness with which they are examined, both clinically and by laboratory means In one outbreak, Visser (1940) examined the CSF of all children and of the nursing staff, he found suspicious changes in all specimens Examination of spinal fluid protein has been used as a means of establishing the diagnosis in contacts who have had a suspicious illness (Andelman *et al.*, 1946)

Infection in laboratory workers and nurses.

Infection has occurred in laboratory workers handling infected cords, stools and other materials (Sabin and Ward, 1941 *a*, Gear and Rodger, 1946, Wenner and Paul, 1947). Wenner and Paul suggest that human material and freshly isolated strains are much more dangerous to work with than "stock" virus, the danger to technicians undertaking the diagnosis of poliomyelitis by laboratory methods is not inconsiderable In our laboratory we insist on the wearing of protective gowns, masks, gloves, and boots Nurses only occasionally develop paralytic or abortive symptoms, although it is probable that a proportion becomes infected and excretes virus in the stool (International Committee, 1932, Gear and Rodger, 1946) An unusual occurrence was the 1934 California outbreak where 11 9 per cent. of hospital employees became infected (Kessel, Hoyt, and Fisk, 1934).

The Carrier

Convalescent carriers are known to be unusual, as the great majority of persons ceases excreting the virus in the stool by two months and in the nasopharyngeal secretion by a few days We can safely conclude that convalescent carriers play no significant part in the spread of poliomyelitis

We use the term "healthy carrier" to refer to persons who have contracted infection, usually by intimate exposure to an abortive or paralytic case, but who show no symptoms of invasion Contacts so exposed who develop symptoms are

sidered under the heading of "The Acute Case," above. Some of these carriers may have had an illness of infection, but no further evidence of invasion of the tissues (Conybeare, 1946). Healthy carriers excrete the virus in the feces for a period similar to that in the acute case, and it can also be found in the nasopharynx. Another type of carrier is the "incubating" or "precocious" carrier. Such a person harbors the virus in feces or nasopharynx several days before the onset of symptoms. In one instance a boy with a positive stool, who later developed the disease, infected 7 contacts in the incubation period (Gear and Mundel, 1946).

In towns and cities each household is daily in contact with the outside world, and it is difficult, therefore, to implicate a carrier. Evidence confirming the rôle of carriers has been obtained, however, by certain workers who have studied the epidemiology of the disease in isolated rural areas where human contacts outside the family are strictly limited, and in island communities. For example, in Sweden, Wickman showed that carriers were responsible for infecting cases on isolated farms. Alder (1937) in Switzerland traced infection to the visit of a carrier. James (1938) traced the introduction of poliomyelitis to an isolated island to the arrival of a ship from an infected area. In all probability the St. Helena outbreak was initiated by the arrival of a healthy carrier among service personnel.

Some workers suggest that the Malta 1942-3 outbreak was initiated by healthy carriers in the services arrived on the Island from the Middle East (Bernard, 1945, van Rooyen and Kirk, 1946).

Conybeare (1946) recorded an instance of poliomyelitis developing in the sister of a boy who had attended a camp where poliomyelitis infection occurred, the boy had no symptoms, and must have been a healthy carrier. Doctors and nurses on their rounds may unwittingly act as carriers of infection (Turner, 1945).

In the Texas 1943 outbreak, temporary carriers were blamed (Bohls and Irons, 1944-5). In S. Africa in 1944-5, cases usually had contacts with a village where poliomyelitis was prevalent, or with persons from such a district (Gear, 1946).

The Mauritius 1945 epidemic afforded evidence of spread of infection by carriers (McFarlan, 1946, McFarlan, Dick, and Seddon, 1946). They found that families with a case had more outside contacts, through school, or by adults working away from home, than families not affected. The percentage of adult males working away from home was significantly higher in those families with a case of poliomyelitis. The curve of incidence of paralysis was "peaked," with a rapid fall. The outbreak was "explosive" and suggested that the disease was one of short incubation and high infectivity in a susceptible population. It was suggested that there was a large carrier wave in adults who became infectious a few days after exposure. A week later there was the smaller wave of paralytic infections. This experience was such as strongly to incriminate healthy adult males as transient carriers. The distribution of cases was so diffuse as to suggest that infection was probably spread by droplets and not by feces.

Healthy carriers can cause infection by means of droplets or feces. As virus is present longer in the feces, and as prolonged contact is usually needed, it seems probable that infection is more usually passed in this way than by droplets.

Infection by Arthropods

Mosquitoes and biting flies.

Various points have been put forward on theoretical grounds to support the suggestion that poliomyelitis may be spread by mosquitoes: the sporadic nature of cases, the supposed rarity of direct contact and of [illegible]
in the [illegible]
(Roser [illegible]
that m[illegible] any part in the transmission of the disease, because virus is seldom present in the blood of the acute case.

Numerous experiments have failed to support the theory. Thus Rosenau and Brues (1913) fed large numbers of *Stomoxys calcitrans* on an infected monkey, and immediately after on a normal animal, the infection was transmitted, but only

point of view of

Clark, Fraser, and

Noguchi and Kudo

(1917) were unable to infect monkeys with *Culex pipiens* by feeding on infected water or monkeys. *Aedes aegypti* fed on an infected monkey did not transmit the infection to a normal animal, and no virus was recovered from the mosquitoes by monkey inoculation (Cornell and Davis, 1939) No evidence to incriminate mosquitoes was found in the 1944–5 S. African outbreaks (Gear, 1946)

Paul (1947 *a*) reports that Mitamura has found virus to survive in *Culex* and *Aedes* mosquitoes, infected by feeding, for about 3 weeks.

Lice, fleas, bedbugs.

Lice have been found incapable of transmitting the virus (Howard and Clark, 1912), as have fleas (Kling, Pettersson, and Wernstedt, 1912). Musgrave (1942) suggested that rats might be naturally infected with the virus, and rat fleas might transmit the infection to man. Howard and Clark (1912) found that in one instance virus was capable of surviving, and apparently multiplying for at least 7 days, infection being transmitted by the bite. Conway and Bigwood (1946) wondered whether cat fleas might act as vectors

Houseflies and blowflies.

The fact that virus can be found so regularly in feces raises the question of whether flies are concerned in the spread of the disease. In some outbreaks flies have been reported as unduly numerous in the neighborhood of cases, and some workers have been inclined to attribute a definite part to the fly in the spread of infection (Henson and Port, 1945, Deeny and McCormack, 1946, Gear, 1946)

Various workers have isolated poliomye

Toomey, Takacs, and Tischer (1941) isola
sewage infected stream

In searching for virus, pools of flies should be separated into species, ground, suspended in saline, lightly centrifuged, and the supernatant etherized and inoculated intracerebrally in susceptible monkeys, a gauze filtrate of the supernatant can be inoculated nasally or abdominally. Both blowflies (*Phormia regina*) and houseflies (*Musca domestica*) can carry virus The blowfly appears to be much the most important fly

Flies collected in nonepidemic periods have not been found infected (Trask and Paul, 1943, Trask, Paul, and Melnick, 1943)

Flies infected with virus from feces obviously may contaminate milk and foods intended for human consumption An important experimental observation in this connection is that of Ward, Melnick, and Horstmann (1945) who exposed food to fly contamination in the homes of cases in the first week. The food was then given to chimpanzees, who excreted virus in their stools An extensive outbreak in a naval training school was thought to be due to contamination of milk by flies that had access to feces (see p. 964)

Flies may contaminate by means of their feet. Further, the virus has been found in feces and vomit deposited by flies within a short time of an infected meal (Rendtorff and Francis, 1943) It is important to know how long infected flies may excrete virus Using the Lansing strain, and *Musca domestica*, Rendtorff and Francis (1943) recovered virus for up to 2 days after a single infected meal, but there was

no evidence of multiplication (see also Bang and Glaser, 1943). Important studies have been reported by Melnick and Penner (1947). They fed the stools of acute cases to the blowfly (*Phormia*). Virus was recovered from the body of the fly as late as 12–16 days after the meal, and from the excreta for 3 weeks. In contrast, when murine and Theiler's strains were fed to *Phormia*, *Phaenicia*, and *Sarcophaga*, the virus was recovered for 5 days only, behaving like inert carmine. It is not yet possible to say if virus multiplies in *Phormia*, but this seems a possibility.

All attempts to recover virus from adult flies developing from larvae infected by feeding were negative (Bang and Glaser, 1943). The important question of *whether virus can survive from season to season in flies* has not yet been answered.

Systematic studies of the fly population at New Haven (Connecticut) have been carried out by Power, Melnick, and Bishop (1943), and Power and Melnick (1945), who found that *Phaenicia sericata* was the dominant fly, representing 80–90 per cent of all trapped. Different species were found to be most numerous in different parts of the season. The peak of the fly incidence preceded the peak of the poliomyelitis epidemic by 4–5 weeks.

Sewage and Latrines

Numerous workers have succeeded in isolating virus from urban sewage in epidemic periods (Paul, Trask, and Culotta, 1939; Levaditi, 1940, Paul, Trask, and Gard, 1940, Weston, 1940, Paul and Trask, 1941, 1942; Kling *et al*, 1942 *a*, *b*; Trask and Paul, 1942 *a*, Gear, Mundel, and Wilson, 1946, Melnick, 1946 *a*, 1947).

Virus was detected in Stockholm sewage 3 months after the end of an epidemic, but not 9 months later (Kling *et al*, 1942 *a*, *b*). It was also found in Johannesburg 3 months after the last case (Mundel, Gear, and Wilson, 1946).

Other experiments conducted at the end of epidemics have proved negative (Paul, Trask, and Gard, 1940, Paul and Trask, 1942, Melnick, 1947)

Sediment from a positive sample stored at 4° C. retained its infectivity for at least 3 months, *suggesting that the virus can survive outside the body for some length of time* (Kling *et al.*, 1942 *a*, *b*).

Mundel, Gear, and Wilson (1946) investigating various stages in the process of purification, detected virus in raw sludge, settled sewage, humus tank effluent, but not in treated sludge or sand filter effluent, the humus tank effluent was used for irrigation. Experimental work has shown that activated sludge can destroy virus under certain conditions (Carlson and McKhann, 1943, Carlson, Ridenour, and McKhann, 1943)

Virus has been recovered from privies used by cases (Howitt, Buss, and Shaffrath, 1942).

Virus has been isolated fairly readily from sewage in epidemic periods usually in the late summer and autumn, and it would seem to be present in much larger amount than can be accounted for by the number of cases (see, e g., Melnick, 1947). A possible explanation is that it can proliferate (Kling *et al.*, 1942 *a*, *b*) There is, however, no evidence that virus can proliferate in association with cultures of various intestinal bacteria, or water or sewage protozoa (Brutsaert, Jungeblut, and Knox, 1946 *b*, Evans and Osterud, 1946) It is much more likely that the large amount of virus present is an expression of its wide distribution in the intestines of healthy contacts of cases, and of the frequency of unreported abortive cases. For example, Melnick (1947) suggested that approximately 6 per cent of the inhabitants of a particular area in New York City may have been fecal carriers

There is considerable significance in these reports of the presence of virus in sewage Infected sewage is a potential source of infection in several ways (1) by gaining access to water supplies, or streams, or lakes used for bathing; (2) by affording a source of infection for flies, (3) by being used for manuring land or cultivating vegetables, or, more crudely, as occurs in the Middle East, for washing fruit and vegetables. There are not as yet any definite reports incriminating sewage

as a source of infection, but certain suggestive observations have been made. Thus in Louisiana in 1928–39 it was noted that small towns where there was a water supply but no sewage disposal, had a higher incidence than rural areas where there were neither, or larger towns where there were both (Casey and Aymond, 1940 *a*, *b*). It was accordingly suggested that the practice of liquefying excreta without removal was a factor in determining incidence.

Prior to the 1942–3 Malta outbreak, human excreta had been used as manure—a common custom in the Middle East at all times. In Mauritius the sanitation was very poor and consisted chiefly of unscreened pit latrines. Irish observers have also attached some importance to privies as sources of infection (Deeny and McCormack, 1946).

Technique of Virus Isolation

The most sensitive of several methods appears to be the following (Melnick, 1947). 1–2 gallons of sewage are allowed to settle in the refrigerator. The supernatant is filtered through gauze, 800 c.c. is treated with 120 c.c. ether, corked and placed overnight in the refrigerator. The next morning to the aqueous phase are added 32 c.c. of normal monkey or horse serum (inactivated at 56° C. for 2 hours), followed by 320 gms. $(NH_4)_2SO_4$, stirring till dissolved. After centrifugation at 2000 r.p.m. for 20 minutes the sediment is resuspended in 20 c.c. distilled water, placed in a cellophane bag and dialyzed against cold running water for 4–6 hours. The dialysate is centrifuged at 4000 r.p.m. for 20 minutes and the supernatant then centrifuged at 39,000 r.p.m. for an hour. The gelatinous sediment is taken up in 1–2 c.c. of 10 per cent. normal monkey serum. Following centrifugation at 18,000 r.p.m. for 20 minutes, to remove aggregated material, the sample is ready for cerebral inoculation in rhesus monkeys.

Water Supplies

As virus occurs so commonly in feces and sewage it is quite possible for it to gain access to water supplies. Although earlier work of Kling and Levaditi (1913 *a*) did not incriminate water supplies, Kling (1928) later supported the theory that the disease could be spread by this means. He stated that the distribution of groups of cases was dependent on water supplies, and that the disease first broke out near the supply in the hills, cases occurring successively as the stream descended. Gard (1938) stressed the importance of increased rainfall, and mentioned a laborer who was alleged to have contracted the disease a few days after drinking water from a ditch.

More recently in western Connecticut, Paul and Trask (1941) also found that the distribution of cases followed a water course. In Alabama, Casey (1945) suggested that sewage polluted waters concentrated by drought were responsible for infection in many sporadic cases in adults. In Sweden, Spaak (1945) blamed infected water supplies for sporadic cases occurring in a small parish, when the water was inoculated in monkeys, suggestive changes were produced. By inoculation of cotton rats, Toomey, Takacs, and Weaver (1945) isolated a poliomyelitis-like agent from a polluted stream.

In the 1945 Mauritius outbreak it appeared possible that ice was responsible in one village out of the numerous areas involved (McFarlan, 1946, McFarlan, Dick, and Seddon, 1946).

There has been considerable discussion as to the amount of chlorine needed to destroy poliomyelitis virus in the presence of organic matter in water. Some workers have found that amounts of free chlorine even up to 50 p.p.m., i.e., much more than are used in public supplies, cannot be guaranteed to inactivate virus in the presence of organic matter in a reasonable period (Kempf and Soule, 1940 *b*, Kessel *et al.*, 1943, Trask, Melnick, and Wenner, 1945). Levaditi, Kling, and Lépine (1931) found that virus was inactivated by 4 gm. Cl_2 per liter in 24 hours in the presence of organic matter.

The observations of Ridenour and Ingols (1946) suggest that chlorine can be

used in amounts that will inactivate virus, and yet give a palatable water. They found in tests with samples infected with the Lansing strain of virus that a free chlorine residual of about 0.2 p.p.m. inactivated virus after 10 minutes, with contact for 30 minutes, 0.1 p.p.m. was enough. A complication is the fact that mixed residuals of chlorine and chloramine are usually present (see Lensen, Rhian, and Stebbins, *1946*, *1947*). They found (*1947*) that at pH 6.85–7.4 virus was inactivated after 10 minutes in all samples with residuals of free chlorine and chloramine, even with as little as 0.05 p.p.m. free chlorine. With increasing pH, virus appeared more resistant to chlorine.

The effect of combined aluminum hydroxide sedimentation and sand filtration on infected water has been studied by Kempf *et al.* (*1942*), who found that 1.16 and 1.5 c.c. of sediment per liter produced a noninfective inoculum.

Ozone appears to be capable of rapidly destroying poliomyelitis virus (Kessel *et al.*, 1943).

The following methods of purification were found to hold back the virus in laboratory tests: sand filtration, aeration; absorption to charcoal, alteration of pH, storage, alum precipitation (Carlson, Ridenour, and McKhann, 1942). The same workers found that ultraviolet light destroyed most of the virus under optimum conditions.

It has been found that virus remains viable in tap water at refrigerator temperature for 100 days (Carlson, Ridenour, and McKhann, *1942*).

It may be said that very few workers believe that water is a usual source of infection, although it may account for a few sporadic cases in country areas. Epidemiological inquiries in numerous outbreaks have consistently failed to incriminate water supplies. The evidence against poliomyelitis being a water-borne disease has been marshalled by Krumbiegel (*1944*) who draws attention to these points. (1) outbreaks bear no relation to individual supplies, (2) outbreaks are not "explosive", (3) there is no association with other evidence of pollution. Similar remarks have been made by Maxcy (*1943*).

Milk

A number of outbreaks has definitely been traced to milk (*International Committee*, 1932). Of these the best known in Great Britain is the 1926 Broadstairs epidemic (Aycock, 1927). In America that in Cortland (New York) recorded by Knapp, Godfrey, and Aycock (*1926*), and that in a Midwest college (Rosenow, *1932*) attracted *much interest. Goldstein, Hammon, and Viets* (*1946*) reported an outbreak among cadets at a naval training school. Within 8 days there were 18 cases of polioencephalitis, and 100 of abortive poliomyelitis. Flies were numerous, had access to feces, and probably infected milk. Dead flies were common in milk jugs. Horstmann (*1946*) has blamed milk on general grounds.

In the majority of outbreaks the onset has been explosive, the cases having consumed milk from one dairy. Infection of the bulk supplies is presumably by nasopharyngeal droplets or feces from a carrier or abortive case employed in handling the milk.

Although poliomyelitis occurs in the chief milk-drinking age groups, and the virus can survive in milk for some weeks (see p. 1002), it is unlikely that the disease is spread by this means except on rare occasions.

Food

The height of the curve of poliomyelitis incidence coincides with that of the harvest of perishable fruits and vegetables, it has been suggested, therefore, that these articles may harbor the virus (Toomey and August, *1933*, Gebhardt and McKay, *1946*). Barber (*1938*) observed 4 cases developing in a single house of a boarding school on the same day, and tentatively suggested that the source of infection was strawberries. From their experience of poliomyelitis and infective hepatitis

(which have certain epidemiological resemblances) in the Middle East, van Rooyen and Kirk (1946) strongly support the importance of uncooked food or vegetables. They state that the consumption of uncooked food and vegetables, however well washed, is an ever present danger in the Orient [illegible]
have been used from time immemorial [illegible]
is to wash vegetabl[illegible]
of the local [illegible]
and officers' [illegible]
ogaster (the fruit fly) in [illegible], 1947).

Scobey (1946) has sug[illegible] changes ascribed to the virus of poliomyelitis may be produced *under the influence* of hydrocyanic acid found in fruit and vegetables.

Extrahuman Sources

It is natural to inquire whether there is any evidence that poliomyelitis may be contracted from nonhuman sources. Lumsden (1942), for example, concluded that infection was spread by infected localities, rather than by human contacts. He blamed hosts such as rats, birds, fowls, and bovines, with fleas, flies, and mosquitoes as vectors

There is no evidence that rats, chickens, pigeons, or larger farm animals harbor poliomyelitis virus, or that ants or cockroaches are infected (Toomey, Takacs, and Tischer, 1943, Gordon, 1945 *b*, Pearson and Rendtorff, 1945 *a*, Pearson *et al*, 1945). Although others have found no evidence that dogs may be infected with virus, Gordon (1945 *b*) reports that the sera of 3/37 animals neutralized the Lansing strain Poliomyelitis virus cannot live or multiply in tomato plants (Toomey and Corum, 1943)

A certain amount of suspicion has been thrown on pigs, who may suffer from a paralytic infection known as Teschen disease (see Ch LXXXI) Histologically this condition is identical with poliomyelitis, and is apparently due to a filtrable virus The highest incidence is in winter The balance of opinion is in favor of the disease being unrelated to human poliomyelitis (Frauchiger and Hofmann, 1941, Gard, 1943 *a*)

There has been some suggestion that paralytic conditions of chickens may be transmissible to man Thus, Preston (1941) observed cases of poliomyelitis, on an isolated farm, who had had no contact with the outside world for several months. He suggested that the infection derived from paralyzed chickens and turkeys, the causal agent may have been transmitted by the chicken louse

The only extrahuman source to be seriously considered is that of rodents, for a number of viruses similar to poliomyelitis, especially Theiler's virus, infect these animals under natural conditions (see Ch. LXXXI) Some human poliomyelitis strains also infect rodents. It is evident, therefore, that poliomyelitis virus is not a unique agent affecting man only, but is closely related to agents producing disease in rodents It has been suggested that human poliomyelitis virus is probably originally derived from rodents (Burnet, 1945 *b*)

Jungeblut and Dalldorf (1943) isolated a rodent-pathogenic virus (MM) from the brain of a house mouse found dead in the home of a fatal case of human poliomyelitis, a somewhat similar virus was found in the brain stem of the human case

Later (1946) they isolated 2 strains falling into the murine poliomyelitis group from a number of house mice trapped in the New York area. These strains were not identical but were closely related Brown and Francis (1947 *a*) found that rat sera might neutralize rodent strains, but failed to isolate virus from rat tissue.

At present, it does not appear to us likely that rodents, or other extrahuman agencies mentioned above, need be seriously considered as a source of infection in poliomyelitis

Conclusions

Certain conclusions can be drawn from the mass of detail available on the subject of the method of spread of poliomyelitis.

1. Evidence is strong that many cases are infected by intimate personal contact with abortive or paralytic cases, or with close contacts of these cases, who may or may not develop symptoms. Child contact is more important than adult contact Feces or less probably droplets may be the actual means of transfer of virus.
2. Some cases are infected by healthy carriers
3. Other cases are definitely precipitated by tonsillectomy and other less well defined traumata, the infection being autogenous.
4. Certain outbreaks have been definitely shown to be due to infected food, such as milk. Unwashed fruit and vegetables are also of importance.
5. Flies may be vectors, and can transmit the virus from feces to food.

PERSONAL PROPHYLAXIS IN POLIOMYELITIS

Following nasal instillation of alum, picric acid, zinc sulfate, or other chemicals, monkeys become insusceptible to nasal applications of virus (see p 986) Field reports of the use of these chemicals in man have been unsatisfactory, probably chiefly because of the rarity with which virus invades by this route.

The United States Public Health Service in 1936 suggested for trial a nasal spray of equal parts of 1 per cent. picric acid and 1 per cent. sodium alum in saline, this was to be applied 3 to 4 times daily in alternate days, and thereafter once weekly Armstrong (1936 *b*) reported the results of a trial of ½ per cent. each of picric acid and sodium alum sulfate dissolved in normal saline. As cases occurred in persons so treated, the effectiveness of the instillations could not be asserted.

Following on Schultz and Gebhardt's (1937) work with monkeys, Peet, Echols, and Richter (1937) recommended zinc sulfate sprays (see also Pentecost, 1937, Abt and Abt, 1938, Hayden, 1938, Schultz, 1938; Shahinian *et al*, 1938, Smith, 1938) Myerson (1938) recommended a 1 per cent. aqueous solution of picric acid Zinc sulfate has been used in Australia (Gutteridge, 1938).

The zinc sulfate spray was given a thorough trial in the Toronto 1937 autumn epidemic (Pentecost, 1937, Tisdall *et al.*, 1937). All spraying was performed in the most efficient manner, but no preventive value was shown

GENERAL PREVENTIVE MEASURES

The safest practice is to assume that cases of poliomyelitis are infective by nasopharyngeal droplets and feces. Accordingly the measures to be followed are similar to those adopted in cases of known droplet-and known feces-spread disease. Numerous authors have discussed the measures to be adopted in the control of poliomyelitis (*Ministry of Health Memorandum 1936*, MacNalty, 1938, 1942, Smith, 1939, Deeny and MacCormack, 1946, Gear and Rodger, 1946, *Medical Officers of Schools Association*, 1946), and their recommendations are broadly as follows.

Notification is practiced in almost all countries, and nonparalytic cases should be as fully reported as paralytic cases It is certain that the figures of incidence published by various countries are too low, because of a failure to report a considerable proportion of nonparalytic cases In America, where the position is probably better than in many other countries, about 70–85 per cent of cases are reported (Collins, 1946). The diagnosis of nonparalytic poliomyelitis is difficult, the tendency is to make the diagnosis with more readiness in the families and contacts of cases, than in persons not known to be exposed A personal canvass of all child contacts will result in cases being detected that would otherwise be missed The question of the Kenny concept is of considerable importance here, for the inclusion of cases showing spasm will result in a considerable increase in notification

Nelson and Aycock (1944) came to the following conclusions on the accuracy

of reporting The lowest percentage of cases reported was in the smaller localities. Cases were less frequently reported in the "off season" than in August, when 90 per cent were reported Cases under 2 and over 16 were often not reported.

Isolation. Cases, either abortive or paralytic, are usually isolated ... s. It should be remembered, however ... e ... s

... ing the spread of t ... Rol

Child contacts, a high p ... are probably excreting virus in the stools, should be quarantined at home for 3 weeks, and thus kept from school and other child gatherings.

It is not usual ... siderable propo ... adult contact w ... close contact with ... other than his c

A ... who q ... for a f

At ... of quarantine the homes should be disinfected

Opinions on the effectiveness of isolat ... tacts are varied For exam ... Australian epidemic

Isolation of the family or community. In rural areas it may be possible to isolate affected families, and a few judicious words to neighbors will discourage visits to a possible source of infection. As regards villages, Gear and Rodger (1946) in N Rhodesia were able to ban visitors to the village affected, so that the chances of the infection spreading elsewhere were minimized

Day schools In rural areas, day schools may usefully be closed, and child contact thereby minimized

In urban areas it is doubtful whether school closure as a general measure is of any value The occurrence of several cases in a class would, however, suggest the advisability of closing this class, and quarantining the children at home, school absentees should be visited, as many may be suffering from abortive poliomyelitis (Payne, 1941)

Residential schools The advice of the Medical Officers of Schools Association (1946) is as follows. (1) If the parents wish to remove the child they can do so if he can be isolated in transit and at home for at least 3 weeks. (2) If secondary cases appear (this is unusual) it may be best to disperse the school for at least 3 weeks. (3) On the occurrence of the first case, the school should be quarantined and all outside games and other fixtures cancelled (4) Strenuous games should be minimized.

Congregation of children. During times of epidemic, gatherings of children of all ages should, as far as possible, be forbidden. Children should not visit cinemas, Sunday schools, meetings of clubs or organizations, or camps If contacts are not quarantined, then they certainly must be prevented from attending these gatherings.

Instruction of doctors and general public The health offic ... all practitioners, health visitors school ... The ... paral ... should ... opera ... out-of ... of personal hygi ... handwashings sh ...

Provision of diagnostic and hospital facilities. It may be as well for the health officer to arrange for the services of an appropriate number of consultants to aid practitioners in diagnosis Laboratory facilities may require to be augmented

Arrangements should be made for the appropriate treatment, usually in an orthopedic hospital, of paralyzed cases, as a therapeutic measure rather than as a means of limiting spread.

Water supplies For additional safety, unchlorinated supplies should be boiled, or otherwise sterilized, during times of epidemic prevalence.

Disposal of excreta. The urine, feces, soiled handkerchiefs and bed linen of cases and contacts should be sterilized, e.g., by carbolization. Where privies and latrines are used, feces must be rapidly and completely covered with earth.

Food. Every attempt should be made to prevent fly contamination of foodstuffs, it may be desirable to boil milk shortly before consumption. Antifly measures should be taken, especially where privies or latrines are used, although large scale experiments using DDT in epidemic areas in the United States have not given a definite answer regarding the value of such procedures (Melnick *et al*, 1947).

Utensils used in eating and cooking should be immersed in chlorinated water before being washed (Deeny and MacCormack).

Operations. No tonsillectomies, adenoidectomies, or teeth extractions should be carried out during a period of epidemic prevalence.

Fatigue. During epidemic periods, children should as far as possible not indulge in strenuous games or occupations.

Bathing Bathing in streams, lakes, or even in chlorinated water in baths should be forbidden, owing to the risk of ingesting feces- or droplet-contaminated water.

CHAPTER LXXXIV

INFECTION OF MONKEYS WITH THE VIRUS OF POLIOMYELITIS

Some Other Suggested Etiological Agents of Poliomyelitis

It is not proposed to dwell in any detail on the numerous theories propounded to explain the etiology of poliomyelitis This has not been considered necessary in view of the present almost universal acceptance of the etiological nature of the virus, but those desiring further information on earlier theories should consult the survey of the International Committee (1932)[1] Two of these theories only will be considered in any detail.

Rôle of streptococci.

As in other disorders in which a virus has been isolated, claims for the rôle of a cultivable coccus have been made, and streptococci have been isolated by a comparatively large number of workers (e g, Mathers, 1916, 1917, Nuzum, 1916, 1917 *a*, *b*, 1918, Nuzum and Herzog, 1916, Rosenow, 1917 *a*, *b*, 1918 *a*, *b*, 1925, 1926, 1928, 1929–30, 1931, 1932 and see below, Rosenow and Gray, 1918, Rosenow and Wheeler, 1918, Hektoen, Mathers, and Jackson, 1918, Wayson, 1918 *b*, Cooper, 1931 *a*, *b*, Coole, 1945)

The main facts elicited by such workers will be briefly summarized Alpha-hemolytic streptococci were isolated from the brain, nervous system, and other parts of the body The organisms were filtrable through Berkefeld filters, and growth could be obtained from the filtrates Anaerobic cultures yielded growths of organisms similar to the "globoid bodies" (see below) The organisms were pathogenic for rabbits and monkeys, and were said to produce a disease similar to human poliomyelitis Antisera obtained from the horse were used in treatment Experimentally, however, these streptococcal antisera behaved in the same way as normal horse sera, and were not able to neutralize the virus (Amoss and Eberson, 1918 *a*, *b*, see also Stewart and Haselbauer, 1928) Precipitin reactions with nasopharyngeal washings were positive in contacts and cases, as well as in a smaller percentage of persons in the infected locality These streptococci were often regarded as one stage in a life cycle, the poliomyelitis virus being held to be the other stage Filtrates of streptococci were not, however, identical with the virus (Richardson and Mellon, 1931–2) Whereas the virus resisted glycerol for several years, the streptococci did not survive much over one year (Long and Olitsky, 1928–9, Rhoads, 1929)

Of recent years, Rosenow has strongly supported the rôle of streptococci in the etiology of poliomyelitis (see, e g, 1937, 1938, 1939 *a*, *b*, *c*, 1940, 1941, 1942, 1943 *a*, *b*, *c*, *d*, 1944 *a*, *b*, *c*, 1945) His main observations are as follows

1 Specific *alpha* streptococci have been isolated from the nasopharynx, stools, and blood of human cases, from experimental animals, water, sewage, flies, and dust

2 The inciting agent of poliomyelitis is represented by interrelated phases of the streptococcus and virus The streptococcus plays the primary rôle in the causation of the disease, the occurrence of epidemics, and the acquisition of immunity The virus is the small filtrable, highly invasive but relatively nonantigenic phase of the streptococcus Pleomorphic diplococci can be found in filtrates of cultures of streptococci, and are considered to be forms of the virus The streptococcus and virus are two forms of the one agent, the streptococcus is not a passive invader, but a part of the infectious process generally attributed to the virus

3 The serum of poliomyelitis patients agglutinates the streptococci Streptococci are also agglutinated by monkey convalescent serum

4 Precipitation occurs between antistreptococcal serum and extracts of nasopharyngeal swabbings or serum of patients

5 A cutaneous reaction has been developed in diagnosis This reaction is elicited on injection of the supernatant of an autoclaved suspension of streptococci The reaction is

[1] References are appended at the conclusion of Ch LXXXVIII, p 1026 et seq

greatest in the early stages of the disease; it disappears gradually on recovery, and abruptly when antistreptococcal serum is given

6 The intracutaneous injection of a specific polysaccharide also elicits a reaction, which is slight at onset but increases in convalescence.

7 Hyperimmune (horse) antistreptococcal serum also gives a skin reaction in infected persons.

8 Filtrates of streptococcal cultures in a special egg medium produce a fatal result on nasal inoculation in mice.

9 Monkeys recovered from infection with streptococci are immune to the human disease. They can also be immunized against the virus by injections of streptococcal vaccines combined with streptococcal antisera.

10. Antigen-antibody reactions specifically related to the streptococcus were demonstrated in the skin and serum of rhesus monkeys during experimental poliomyelitis Injections of antistreptococcal serum caused the skin and serum reactions to become negative.

The reactivity of the skin and serum of monkeys was demonstrated in 4–24 hours after intracerebral injection of killed poliomyelitis and arthritis streptococci, it disappeared in 48 hours

11. Antistreptococcal serum is of benefit in treatment of the human disease

Many workers have noted that animals injected with streptococci develop purulent lesions without paresis or other evidences of poliomyelitic infection (see, e g, Bull, 1917, Smillie, 1918; Wayson, 1918 *b*; Long, Olitsky, and Stewart, 1928, Fairbrother, 1929). It is not generally believed that streptococci play any important part in the etiology of poliomyelitis.

Globoid bodies.

A number of authors reported that opalescence due to small spherical structures or "globoid bodies" could be noted in rich culture media (such as ascitic fluid with rabbit kidney), inoculated with cerebrospinal fluid or brain tissue, and incubated anaerobically (Flexner and Noguchi, 1913 *a, b;* Amoss, 1914, 1917, Flexner, Noguchi, and Amoss, 1915, Tsen, 1918, Krauspe, 1928, Long, Olitsky, and Rhoads, 1930).

Small colonies consisting of clumps of bodies could be found in semisolid ascitic agar These globoid bodies were filtrable and apparently, on occasions, induced poliomyelitis in monkeys even after considerable dilution Despite certain of these results which appeared suggestive, it is not generally believed at the present day that the globoid bodies can represent the virus of poliomyelitis Logrippo (1936) investigated the whole position, and the following are among his more important findings (*a*) the opalescence in culture media thought to be specific for virus growth can be obtained in the complete absence of virus, (*b*) micrococci also develop in these tubes without the presence of virus, (*c*) forms resembling globoid bodies, and consisting of tissue lipoids, can be produced by the establishment of an electric field in the medium

Both streptococci and globoid bodies are now almost universally acknowledged to be without significance in the etiology of poliomyelitis, which is held to be caused by a specific filtrable virus, whose properties will now be described

HISTORICAL INTRODUCTION

Landsteiner and Popper (1908, 1909) were the first workers to record successful transmission of the infection to monkeys This work was rapidly confirmed by Flexner and Lewis (1909 *a, b, c*), Knoepfelmacher (1909), Leiner and Wiesner (1909); Landsteiner and Levaditi (1909 *a, b*), and many others It at once became evident that the causal agent was a specific filtrable and noncultivable virus Since the early days, large numbers of strains of virus have been isolated in all parts of the world and many have been passaged continuously since the isolation without fundamental change of character No purpose [illegible] more than a few of the later isolations of virus (e g [illegible] Romer, 1912, McIntosh and Turnbull, 1913, A[illegible] Flexner, 1932; Brodie, 1934–5 *d*, Kessel, Hoyt, and Fisk, 1934, Trask and Paul, 1936, Kessel *et al.*, 1936–7, Howitt, 1937 *a*, Sawers, 1938). Many of the early workers injected the virus intraperitoneally, intracerebrally, or by both routes combined

It soon became evident that monkeys are susceptible to infection by almost every possible route.

THE STANDARDIZATION OF VIRUS INFECTIVITY

It is clearly necessary to attempt some sort of biological standardization, for example, analogous to the MLD of various toxins, in the case of the virus of poliomyelitis. This has been carried out by Brodie (see 1932 *a*, 1933 *b*) who has formulated a standard "m c p." (minimal completely paralyzing) dose of virus, this is the smallest quantity of virus-containing tissue which will produce a complete and rapid paralysis in a monkey weighing 2.5 to 4 kilos within 13 days after intracerebral inoculation Less than 1 m c p. dose may cause infection, but only after a prolonged incubation period. Conversely, large amounts of virus (e g, 1,000 m c p. doses) may produce paralysis in as short a time as 3½ to 5 days, but doses up to 80 m c p. do not cause infection in any shorter space of time than the normal 7 to 14 days (Brodie, 1933 *b*). Madsen and Jensen (1936) have made use of the m c p method of estimating virus infectivity in their preparation of a standard poliomyelitis antiserum (see p. 1023).

It has been shown that pooled cords, ground up and mixed with 50 per cent. glycerol, retain a constant infectivity for at least 12 months (Kramer, Schaeffer, and Park, 1934).

When using mouse-pathogenic strains, titers can be expressed on the basis of calculating 50 per cent mortality end points

DIFFERENCES IN INFECTIVITY OF STRAINS

It is not possible to isolate virus from all cases of poliomyelitis by monkey inoculation, and probably human strains vary in their ease of adaptation to this host (see, e g, Flexner and Amoss, 1924 *b*) Once established, strains may vary widely in their infectivity Thus, Kessel, Moore, and Pait (1946) titrated 4 strains intracerebrally in *M mulatta* and obtained the following titers MV and Le—1 10,000 McK—1/1,000, Ba—1 '100. These differences were probably inherent, and not associated with the number of passages Recently isolated strains show a high degree of infectivity on intradermal injection, while passage strains are less regularly infective by this route (Trask and Paul, 1938)

The MA strain has undergone fluctuations in virulence At first feebly pathogenic to monkeys, it later increased in virulence, and became virtually fixed for about 3 years Then it again became of diminished virulence (Flexner, Clark, and Amoss, 1914 *a*) After treatment with glycerol, its former virulence was restored (Flexner and Amoss, 1914 *a*) A somewhat similar loss of virulence was reported with the LP strain which had been passed since 1910 (Levaditi and Hornus, 1932)

SPECIES SUSCEPTIBLE TO THE VIRUS

The majority of investigators has used rhesus monkeys (*Macaca mulatta*) and found them satisfactory, but numerous other varieties are at least as, if not more, susceptible The chimpanzee (*Pan satyrus*) is markedly susceptible Cynomolgus (*M irus*) monkeys have been quite widely used, Burnet, Jackson, and Robertson (1939 *b*), in titration experiments, found cynomolgus and rhesus monkeys equally sensitive to a passage strain (MV), but when a fresh strain (Mar) was used, the cynomolgus monkeys were far more susceptible Howe and Bodian (1947 *b*) using cerebral inoculation, found rhesus and African green monkeys equally susceptible Cynocephalus, cercocebus, cercopithecus, *M nemestrinus*, and *Papio babuin* monkeys, have all been found susceptible

The following named species of monkey belonging to the cebus, cercocebus, cercopithecus, erythrocebus, macacus, and papio families are among those that have been found suitable in recent years, and can replace rhesus monkeys

Species of Monkey	*Reference*
Cebus capucina (S American ring-tail)	Melnick and Paul (1943) Melnick (1946 *b*)
Cercocebus fuliginosus	Schabel and Gordon (1947)
Cercocebus torquatus	Findlay, Anderson, and Haggie (1946)
Cercopithecus aethiops sabaeus *C. aethiops centralis* *C. aethiops pygerythrus* (vervet)	Trask and Paul (1941 *b*), Paul, Havens, and van Rooyen (1944), Finlayson and Wicht (1945), Gear, Yeo, and Mundel (1945), Melnick and Ward (1945), Melnick (1946 *b*)
	Findlay, Anderson, and Haggie (1946)
Cercopithecus cephus (African mustache)	Ceccaldi (1943); Melnick and Paul (1943), Melnick (1946 *b*)
Cercopithecus diana roloway	Findlay, Anderson, and Haggie (1946)
Cercopithecus griseoviridis (grivet)	Paul, Havens, and van Rooyen (1944)
Cercopithecus mona mona	Melnick (1946 *b*)
Cercopithecus nictitans	Ceccaldi (1943)
Erythrocebus patas (hussar)	Paul, Havens, and van Rooyen (1944), Findlay, Anderson, and Haggie (1946)
M mordax	Melnick (1946 *b*)
Papio hamadryas (Abyssinian baboon)	van Rooyen and Morgan (1943); Paul, Havens, and van Rooyen (1944)
Papio papio	Findlay, Anderson, and Haggie (1946), Schabel and Gordon (1947)

The spider monkey (*Ateles ater*) is not susceptible (Mackay and Schroeder, 1935–6), nor is the common marmoset (Grossman and Kramer, 1936–7)

FACTORS PREDISPOSING TO INFECTION

Following ligation of the carotids, monkeys are more susceptible to inoculation (Schwab, Woolpert, and Hudson, 1943) It has been suggested that hypoglycemia may be an important factor determining susceptibility Monkeys subjected to exhausting exercise in the incubation period develop severer paralysis than controls, chilling has the same effect (Levinson, Milzer, and Lewin, 1945) The incidence and severity of paralysis is significantly greater in monkeys inoculated in the summer (Moore *et al.*, 1942, Moore and Kessel, 1943, Levinson, Milzer, and Lewin, 1945) Trauma applied to the limbs in the incubation period does not influence the severity or location of paralysis (Levinson, Milzer, and Lewin, 1945)

The rôle of vitamins has been investigated It has been claimed that virus spreads more readily along nerves in vitamin D-deficient monkeys (Toomey, 1940 *a*), although the nerves of rachitic animals show no difference in their ability to absorb virus *in vitro* (Toomey, Tischer, and Takacs, 1942) However, Sabin *et al* (1941) found no evidence that the ability of virus to reach the CNS after sciatic inoculation depended on vitamin D nutrition Rhesus monkeys deficient in thiamine did not exhibit increased susceptibility (Clark *et al.*, 1945) Rhesus monkeys suffering from a chronic deficiency of folic acid showed an increased resistance to inoculation, those with an acute deficiency did not exhibit this resistance (Lichstein *et al*, 1946)

As regards the possible influence of endocrine factors, it has been found that uncastrated female rhesus monkeys given theelin nasally show a normal degree of susceptibility to intranasal infection (Schultz, 1941) Normal monkeys treated with stilbestrol showed no increased resistance, treated castrates, however, showed a

marked increase in resistance to nasal instillation (Curley and Aycock, 1946). Sexually immature monkeys develop a severer disease than older animals (Moore *et al*, 1942; Moore and Kessel, 1943).

ROUTES OF INFECTION WITH THE VIRUS

1. Intracerebral

Virus is usually inoculated with a ¾ inch needle into the cerebral hemisphere on one or other side. Bodian and Howe, however, recommend inoculation of 0.4 c.c. into both thalami. The skull is exposed, and a hole drilled ⅛ inch to either side of the midline, just caudal to the frontoparietal suture A 24 gauge 1 inch needle is used to inject (see Morgan, Howe, and Bodian, 1947).

This route is the most certain in its results (e g, Landsteiner and Levaditi, 1909 *b*, Flexner and Lewis, 1909 *a*, 1910 *d*, McIntosh and Turnbull, 1913). Paralysis usually develops after 7 to 10 days, but if this does not occur, the monkeys can be reinoculated, when an accelerated response takes place with the rapid development of the characteristic syndrome (Flexner, 1931 *a*). If monkey strains are inoculated together with autolyzed mouse brain, a fulminant infection is said to result (Milzer and Byrd, 1947).

After intracerebral injection, the virus increases locally for a short time (Jungeblut, 1930–1 *d*), but soon leaves the site of inoculation and spreads to other parts of the brain The paths taken by the virus, and the changes produced, have been studied in particular by Fairbrother and Hurst (1930), whose work will now be described

For the *first* 2 days there is some localized nerve cell destruction at the site of inoculation, the meninges over the cerebral hemispheres show an infiltration of the pia with lymphocytes

By the *3rd and 4th days* the homolateral thalamus has become involved, and sometimes the midbrain and pons, but never the cord.

By the *5th day* the cerebral cortex shows the following changes Meningitis is irregular in intensity and distribution, and is associated with perivascular infiltration of the superficial cortical vessels There are also deeper perivascular and more diffuse infiltrations, most marked on the inoculated side, and unassociated with any overlying meningitis, these deeper lesions are pronounced in the vicinity of the site of inoculation The homolateral globus pallidus, anterior perforated space, and amygdaloid nucleus all show perivascular infiltrations. The homolateral thalamus is severely involved and the contralateral to a lesser extent No definite chain of lesions can be demonstrated connecting the site of inoculation with the basal nuclei Nerve cell lesions are now evident in the stem and spinal cord, but are quite unassociated with interstitial or meningeal changes

By the *8th day* the changes have approached those described as characteristic of the declared disease with lesions widespread in the cord and brain stem and much severer than those noted on the 5th day (Hurst, 1929, see p 991). The lesions elsewhere in the central nervous system, on the other hand, have not increased much in severity

Fairbrother and Hurst conclude from their study that the spread of the virus is essentially axonal, and that the primary changes are those of nerve cell destruction, and are not meningeal or vascular In this assumption they have the support of Pette, Demme and Kornyey (1932), and Luhan (1937) Howe and Ecke (1937–8 *a*) have found definite evidence of the spread of virus by decussating paths, they injected monkeys in certain selected areas of the brain and studied the situations where paralysis first appeared Thus, after injection of the visual cortex, uncrossed paralysis developed in 50 per cent of cases, after injection of the premotor area, crossed paralysis developed in a little over 50 per cent of cases, and, finally, after injection of the motor area, crossed paralysis appeared in almost all monkeys They

concluded that these results were what would be expected if the virus spread to the cord via definite nerve tracts.

Bodian and Howe (1939) reported that, in the prodromal stages after intracerebral inoculation, the distribution of lesions suggested that the pathway from the motor cortex to the lower centers was via the globus pallidus, zona incerta, field of Forel, substantia nigra, and tegmentum of midbrain, and not by the [illegible] t cortical-spinal route.

[illegible]

of [illegible] [illegible] intracerebral [illegible] such animals, the characteristic histological changes, as well as the presence of the virus itself, can only be detected in the proximal segment of the cord. Although the main spread of virus thus seems to be axonal, Schaeffer and Muckenfuss (1938) showed that after intracerebral injection, virus enters the cerebrospinal fluid more readily if long needles are used, and the inoculum is large.

2. Intraspinal

The virus of poliomyelitis may be injected intraspinally or intracisternally, and this is a reliable route of infection (see, e.g., Flexner and Lewis, *1910 c*; Clark and Amoss, 1914, Hurst, 1932). After such intrathecal injection, the virus is demonstrable in the cerebrospinal fluid for at least 5 days, but is usually absent at the time of onset of paralysis (Clark and Amoss, 1914; Hurst, 1932). Howe and Peele (1939) found that infection developed on intrathecal injection only if there was pial injury

With regard to the exact route whereby the virus enters the central nervous system from the cerebrospinal fluid after intrathecal injection, there is some difference of opinion. On the one hand, Clark and Amoss hold that the virus first causes involvement of the pia-arachnoid, and then probably spreads in along the perivascular spaces to involve the parenchyma of the cord and brain.

Hurst, on the contrary, holds that the virus enters by the ependymal lining of the 4th ventricle. He did not find that the site of severest meningitis necessarily corresponded either to underlying parenchymal lesions or to the distribution of the virus, as would be expected if infection spread in from the meninges He found lesions typical of poliomyelitis in the vicinity of the 4th ventricle, in certain cases where the animals died rapidly, there was widespread necrosis of nerve cells in the pons and medulla, thus clearly suggesting that this area was the portal of entry. Entered through the 4th ventricle, the virus presumably spreads by nerve tracts to the cord and motor cortex, as Hurst regularly detected it there during the period of paralysis.

Animals can be infected by inoculation into the lumbar cord (Melnick, Horstmann, and Ward, 1945).

3. Intraneural

The sciatic and radial nerves of the monkey have been injected by various workers and infection found to result with some regularity, especially if the nerve is traumatized by the needle (e.g., Flexner and Lewis, 1909 *b*, 1910 *c*, Landsteiner and Levaditi, 1909 *b*, Leiner and Wiesner, 1910 *a*, Hurst, 1930, Toomey, 1934–5 *b*, *d*, Bodian and Howe, 1940 *a*, *b*, Sabin *et al*, 1941, Sabin and Ward, 1942 *a*, Howe and Bodian, 1942 *a*, Toomey and Takacs, 1942) The nearer the nerve injected to the central nervous system, the quicker is the onset of paralysis (Demme, 1930).

If large nerves are cut, and virus is injected intravenously, poliomyelitis may develop, the virus presumably enters the cut extremities, and spreads along the nerves to the central nervous system (Lennette and Hudson, 1936 *a*) Sabin and Ward (1942 *a*) studied the distribution of virus after sciatic inoculation They found none in the nasal secretion, olfactory bulbs, nasal mucosa, tonsils, salivary

glands, adrenals, superior cervical sympathetic ganglia, abdominal celiac ganglia, and small intestines. Virus was found only in the cord

Hurst (1930) carried out an extensive investigation into the pathogenesis of poliomyelitis following intrasciatic injection. He found that monkeys could be infected regularly if the needle point was moved about to traumatize the nerve. After such an injection he found the following course of events (1) The virus spreads centripetally rapidly, although if a section intervening between the site of inoculation and the central nervous is removed within 24 hours, no infection results. (2) Virus is first demonstrated in the lumbar cord, and paralysis appears first on the inoculated side (3) Next, virus is demonstrable in the leg area of the motor cortex, first contralaterally; later, a decussating mechanism comes into play, the virus being found homo [illegible]
the cervical cord and ir [illegible]
may contain virus, but [illegible]
similar studies in a later [illegible]
not demonstrable in the cerebrospinal fluid in the presymptomatic period.

As regards the histological features in monkeys injected by the sciatic route, a number of characteristic changes occurs. In the inoculated nerve itself there is an inflammatory reaction, but only at the site of inoculation, the bulk of the nerve [illegible]

[illegible] but soon spread to the opposite half, and upward to the thoracic and cervical portions, as well as the motor cortex During the spread of the lesions, it is possible to observe that nerve cell degeneration is the primary change, and that the meningeal reaction is only secondary Eventually, lesions typical of established poliomyelitis can be found at all levels of the cord and midbrain With regard to the rest of the central nervous system, there is a somewhat unevenly distributed meningitis over the hemispheres, probably caused by the actual presence of virus in the cerebrospinal fluid, this meningitis bears no relationship to cellular foci or perivascular infiltration in the subjacent tissue.

Toomey (1933–4 *a, c*) has suggested that, following sciatic injection, virus spreads to the central nervous system by the sympathetic nerves He carried out transection of the cord in the thoracic region, and then injected virus into the sciatic nerve Both virus and typical lesions were discovered later in the cervical cord

Howe and Bodian have carried out extensive studies on the pathogenesis of infection after sciatic inoculation (see 1942 *a* for a general presentation of their work). In these experiments they infected the nerve by cutting with sharp scissors, and moistening the central end with virus Symptoms develop in 4–6 days (Bodian and Howe, 1940 *a, b*, 1941 *b*)

1 It would not appear that virus actually multiplies in the nerve, and an infective dose can reach the CNS by a large number of fibers in contact for a short time, or fewer fibers in contact for a longer time (Bodian and Howe, 1941 *b*).

2 The rate of progression in the nerve is approximately 2.4 mms. per hour (Bodian and Howe, 1941 *c*)

3 This result is not altered by previous section or two nerve freezes peripheral to the point of inoculation, 2–6 weeks before the inoculation (Bodian and Howe, 1941 *b*)

4 Inoculation of the nerve, a few moments after freezing at a point central to the inoculation site, does not produce infection The momentary freezing causes degeneration of the nerve fibers peripheral to the point frozen, but does not interrupt the physical continuity Clearly, therefore, living fibers are necessary for the transmission of the virus (Bodian and Howe, 1941 *b*).

5. In other experiments, the nerve was frozen at a single point, and the fibers allowed to regenerate several centimeters into the peripheral segment. The peripheral segment was then cut and inoculated Infection resulted only if regeneration had proceeded for at least 2 months (Bodian and Howe, 1941 *b*).

6. In these experiments, the nerve fibers were successively interrupted by transection or freezing at 2–3 week intervals at least 3 times. Inoculation of the central stump, where the nerve fibers are anatomically intact, did not produce infection About 2 months after the last transection, inoculation of the central stump proved positive. Evidently, metabolic changes induced in neurons or axons during regeneration under these conditions, prevent infection of fibers which are intact as regards vascular supply, lymphatic connections, connective tissue sheaths, Schwann and myelin sheaths. It is, therefore, highly likely that it is the protoplasm of the axon that transmits the virus, but cannot do this in the presence of metabolic disturbances consequent upon regeneration (Bodian and Howe, 1941 *b*).

Bodian and Howe have thus shown conclusively that poliomyelitis virus, after inoculation of the sciatic nerve, spreads to the neurons in the CNS by the axon fibers.

4. Intra-ocular

Injection into the anterior chamber secures infection (e g, Landsteiner and Levaditi, 1909 *b*). Injections into the vitreous also yield positive results (Flexner and Amoss, 1914 *b*) Burnet, Jackson, and Robertson (1939 *a*) have shown that rhesus monkeys are susceptible to inoculation in the posterior chamber with the MV strain. Bilateral ptosis is the usual initial symptom The most intense changes are found in the midbrain, especially in the region of the oculomotor nuclei The virus probably trave

bodies and superior

nerves In experimer

tory changes in various parts of the brain stem, but the 3rd nerve nuclei were not visibly affected, these animals were susceptible to the MV strain and another strain Mar.

5. Intranasal Instillation

Intranasal instillation was employed by Flexner and Lewis (1910 *d*) and Leiner and Wiesner (1910 *a*), and has always proved popular. Pledgets of wool soaked in virus and inserted into the nostrils are also used It is frequently noted, however, that single applications may fail to "take," though repeated injections are more successful *Strains vary greatly in the readiness with which infection* develops Thus, using Aycock's strain, not over 50 per cent. were found to develop infection, while with Flexner's MV strain, 77 per cent. became infected (Schultz and Gebhardt, 1932–3) These workers increased the percentage of "takes" to 95 per cent. by washing the nasal passages with M/15 phosphate solution (pH 5) previous to the actual instillation. The value of this method has been confirmed (*Hudson, Lennette,* and Gordon, 1936, and others) Howe and Bodian (1942 *a*) recommend inoculation with a pipe cleaner The trauma caused by rubbing causes the infection to progress more rapidly. More recently these workers recommend suspending the anesthetized animal with head hanging over the edge of a table, and inoculating directly onto the olfactory area.

Another reason for occasional failure to infect was brought forward by Flexner and Amoss (1920), who showed that the nasal mucosa and secretions might destroy the virus, this poliocidal substance was unrelated to serum viricidal antibody.

Flexner ... central

nervous sys ... ro-

ducing an ... ny

difference in the result of intranasal inoculation according as to whether the monkeys were kept cold or warm

Distribution of virus after nasal instillation. Earlier workers showed that virus could still be demonstrated in the mucosa for up to 4 days after inoculation (Flexner and Amoss, 1920, Faber and Gebhardt, 1933). This question was studied by Sabin and Olitsky (1938), who found that virus disappeared in 4 hours or less from the mucosa, but could be found at 96 hours. They used a strain passaged by the nasal route, which may behave differently.

After inoculation, the virus ascends and can be demonstrated next in the olfactory bulbs, tracts, and lobes (Levaditi and Landsteiner, 1910 *d*, Flexner and Clark, 1912–13, Sabin and Olitsky, 1938). Virus does not ascend, apparently, to the central nervous system in immune monkeys (Sabin and Olitsky).

A careful estimation of the progress of the virus from the nasal mucosa to the cord and other parts of the central nervous system was carried out by Faber and Gebhardt (1932–3, 1933). (*a*) Three days after inoculation the virus could not be found in the homolateral olfactory bulb or elsewhere. However, later Sabin and Olitsky (1938) found virus in the olfactory bulbs 72 hours after instillation. (*b*) By the 4th day the homolateral bulb contained virus and (*c*) by the 5th day the contralateral bulb also. The virus was found elsewhere only in the left hypothalamus and medulla. (*d*) By the 6th day it was present in both thalami in addition. (*e*) By the 7th day it had reached the cervical and lumbar cords and spinal ganglia. The rapid spread of virus was also emphasized by Kramer and Parker (1932–3), and Schultz and Gebhardt (1939).

Faber (1936–7) carried out an experiment which threw further light on the progression of the virus through the nervous system He injected monkeys with trypan blue, and later carried out nasal instillation After 3 days, dilatation of blood vessels in the diencephalon was detected, after 6 days, blue staining (indicating vascular dilatation) occurred in the brain stem, gray matter of the cord, and also in areas in the precentral gyrus and hippocampus. This work clearly demonstrated that by 6 days the virus had reached the cord, leaving a trail of lesions In a later publication (1938), he reported on this work in greater detail In the 1st stage after intranasal inoculation, lesions were found in the olfactory bulbs, and there was fever In the 2nd stage there was a marked and severe reaction in the brain stem down to the medulla, and the secondary olfactory centers were implicated In the 3rd stage there was increased involvement of the medulla, and involvement of the cord was first noted In the later stages the lesions were localized in the cord—sometimes unilaterally Faber concluded that certain nerve cells must be capable of complete recovery, after transmitting the virus Distribution such as that just described can best be explained by postulating that the virus spreads by the olfactory neurons There is strong proof that this is the path, for it has been shown that following interference with the olfactory nerves, either by surgical division or by nasal instillation of chemicals, infection does not follow nasal inoculation Arrived at the brain, the path of spread is probably by the olfactory relays and formatio reticularis (Faber, 1933)

pre;
per
from these areas (viz, the olfactotegmental, hypothalamotegmental, and the periventricular bundle of Schutz) They found no evidence that the spinothalamic tract was a pathway of spread

Virus does not enter the lymph after nasal inoculation (Yoffey and Drinker, 1939)

The histology of the olfactory bulbs These delicate structures are easily torn off and lost in removal of the brain, thus accounting for the lack of information on their histology Sabin and Olitsky have studied this question thoroughly

(1936–7). Four days after intranasal inoculation, definite lesions are present in the bulb. The outer layer of fibers and the subjacent glomerular layer show infiltration with polymorphs and lymphocytes, there is advanced degenerative change present in many of the large mitral cells, with some neuronophagia. It was noted that although virus may be inoculated bilaterally, only one bulb may show these lesions. If the animal fails to succumb, the lesions probably heal quickly. No such changes develop in the bulbs of immune monkeys, nor of normal monkeys inoculated by routes other than the nasal.

Accelerated response after nasal instillation. Generally speaking, paralysis develops 7 to 10 days after inoculation. Should this fail to eventuate, the instillation may be repeated, when fever and paralysis promptly develop (Flexner, 1933 *a*, Elford, Galloway, and Perdrau, 1935). It has been suggested that Draper's dromedary type of the human
due to reinfection, with
pleocytosis and globulin
nasal inoculation, even in immune animals.

6. Inhalation

Rhesus and cynomolgus monkeys can be readily infected by exposure to droplet nuclei of atomized virus in a suitable chamber (Faber and Silverberg, 1941, 1942, Faber, Silverberg, and Dong, 1943 *a*, 1944). Infection is usually contracted by the olfactory route Further experiments were performed where in some animals the olfactory mucosa was treated with zinc sulfate, to exclude transmission of virus by the olfactory pathway. Infection developed in a few of those treated The symptoms and histological examination suggested that virus had entered by the lower respiratory mucosa in the rhesus monkeys, and by the oropharynx in the cynomolgus monkey. In cynomolgus monkeys, the usual route followed was by the afferent fibers of the trigeminal to the gasserian ganglion, and thence to its central connections in the pons and medulla, the 9th and 10th nerves might also be concerned Less commonly, the pathway consisted of the sympathetic fibers of the nose or nasopharynx into the cervical sympathetic ganglia, and thence to the upper thoracic cord Faber, Silverberg, and Dong concluded that infection by inhalation occurred with greater ease than by ingestion.

7. Subcutaneous

Poliomyelitis virus can be found in the regional lymph nodes following injection by this route (Flexner and Lewis, 1910 *a*, *c*) Further, generalization may occur with the development of typical paralytic symptoms (Flexner and Lewis, 1909 *c*, 1910 *c*, Melnick and Paul, 1943)

8. Dermal

Earlier workers failed to transmit the infection by this route (see, e g., Rhoads, 1930). Trask and Paul (1936) found that a fresh strain (Wfd) was infective by this route (see also 1938, 1942 *b*) Other workers have also found that freshly isolated strains, e g, the Sacramento and Mar, may possess this property, but become noninfective on passage (Howitt, 1937 *a*, Burnet, Jackson, and Robertson, 1939 *b*, Burnet and Jackson, 1940, Stimpert and Kessel, 1940, Melnick and Paul, 1943) German and Trask (1938) found that the Wfd strain was still infective when inoculated in areas of skin apparently deprived of nerve supply After inoculation by this route, chimpanzees develop a subclinical infection and excrete virus in the stool (Melnick and Horstmann, 1947)

9. Intravenous

The intravenous route was used by a number of the earlier investigators (e g, Flexner and Lewis, 1909 *b*, 1910 *c*) It never gives very satisfactory results unless an

overwhelming dose is injected (Clark, Fraser, and Amoss, 1914). It has been estimated that to cause infection by intravenous injection, a dose 1,250 times more than that needed for intracerebral injection is necessary (Flexner and Amoss, 1914 *a*). After intravenous injection the virus is demonstrable in the blood for at least 72 hours At the time of onset of paralysis, however, it may have disappeared (Clark, Fraser, and Amoss, 1914); then it can be found in the spleen, marrow, and perhaps spinal ganglia (Flexner and Amoss, 1914 *b*). Animals are still susceptible if the olfactory route is blocked by section or application of zinc sulfate to the mucosa (Rasmussen and Clark, 1940).

Whether virus reaches the central nervous system or not, depends on the integrity of the *blood-brain barrier*. It was shown by Flexner and Amoss (1914 *b*, 1917 *b*) that intravenous injection resulted in paralysis more regularly if the meninges were irritated aseptically by intraspinal injections of serum, saline, or by causing some hemorrhage into the cerebrospinal fluid. Under such conditions, the intravenous infecting dose had only to be 50 times the intracerebral (instead of over 1,000) A similar result can be achieved by a previous intracerebral injection of starch, which serves to "fix" the virus in the central nervous system (Lennette and Hudson, 1936 *a*) Virus was demonstrated in the cerebrospinal fluid, when passage had been facilitated by serum injections, after 3 to 4 days, it persisted till the onset of paralysis (Flexner and Amoss, 1914 *a*).

The state of the blood-brain barrier in experimental poliomyelitis induced by various routes of infection has recently been investigated Thus, using intravenous injections of acid fuchsin and congo red, it was shown that the dye appears in the gray matter, due presumably to a somewhat increased vascular permeability in this area (Harmon and Krigsten, 1937) A slight increase in nitrate (Lennette and Reames, 1937, 1938), but a more definite increase in sodium bromide (Lennette and Campbell, 1937, 1938), has been noted in the CSF after intravenous injection

to the brain can be neutralized in the cerebrospinal fluid by intraspinal injections of immune serum (Flexner and Amoss, 1914 *b*) (Later papers which have con-

permeability are those of Kasahara,

Campbell, 1939, Lennette, Campbell,

The histological appearance of the central nervous system of monkeys injected intravenously differs somewhat from that following other routes of infection, thus the perivascular cuffing is more widespread and there is lymphocytic infiltration of the choroid plexuses (Flexner and Amoss, 1914 *b*).

10. Gastro-intestinal

Numerous workers have failed to infect monkeys orally (Landsteiner and Levaditi, 1909 *b*, Flexner and Lewis, 1910 *c*, Clark, Schindler, and Roberts, 1930, Lennette and Hudson, 1936 *b*, Rasmussen and Clark, 1940, Howe and Bodian, 1947 *b*).

Although rhesus monkeys are difficult to infect orally there is no doubt that some other monkeys (especially cynomolgi) and chimpanzees can be infected by certain strains administered by various methods

1. Simple swabbing of the pharynx with the unadapted Mar strain proved effective (Burnet, Jackson, and Robertson, 1939 *b*, Burnet, 1940)

2 Application to the tongue may rarely produce infection (Faber, Silverberg, and Dong, 1943 *a*) Insertion into the esophagus proved negative (Faber and Silverberg, 1942)

3 Animals may sometimes contract infection, and excrete virus in the stool, by drinking infected water, by eating infected cord, or foodstuffs such as bananas,

milk, and butter infected with virus (Kling, Levaditi, and Lépine, 1929, Levaditi, Kling, and Lépine, 1931; Saddington, 1931–2; Levaditi, Kling, and Hornus, 1933, Vignec, Paul, and Trask, 1939, Burnet, 1940; Trask and Paul, 1941 *b*, Howe and Bodian, 1941 *c*; Bodian and Howe, 1945 *b*). Melnick and Horstmann (1947) found that chimpanzees developed a subclinical infection and excreted virus in the stools

4. Leiner and Wiesner (1910 *a*) found that infection developed after ingestion, if the gut was paralyzed with opium

5. Horstmann *et al.* (1947) infected 2/7 baby rhesus monkeys by feeding with the rodent-adapted Ph and Y-SK strains Both animals developed paralysis, in the case of the animal infected with Ph, virus was recovered from the lymph nodes, wall of small intestine, heart muscle, spleen, and adrenals

Attempts have been made to see whether the upper or lower part of the alimentary system is the more susceptible. Using infected enemata, Faber, Silverberg, and Dong (1943 *a*) failed to produce infection In other experiments, they gave virus in fat-covered capsules that could be absorbed only from the gastro-intestinal mucosa; no infection resulted.

Other workers, however, have succeeded in infecting by stomach tube, rectal tube, or by inoculation into a loop of bowel (Kling, Levaditi, and Lépine, 1929, Levaditi, Kling, and Lépine, 1931, Clark, Roberts, and Preston, 1932, McClure, 1943). The lower part of the alimentary system is, therefore, susceptible, but there seems little doubt, from the experiments of Faber, Silverberg, and Dong (1943 *a*), and other observations, that the upper part of the alimentary tract, i e, the mouth, pharynx, and esophagus, is more vulnerable.

Investigations have beer [illegible] CNS after ingestion, administration by [illegible]
(1941 *c*, 1942 *a*, *b*) infectec [illegible]
thus excluding the olfactory pathway There was a good correlation between the development of bulbar paralysis and administration by the oral route. Virus probably reached the CNS by the 5th and perhaps the 9th nerve. After administration by stomach tube, the celiac and sympathetic ganglia were first involved, then the spinal cord After pharyngeal swabbing, the severest changes in the brain stem were found to be in the nuclei of the cranial nerves in the dorsal region of the medulla (Burnet, Jackson, and Robertson, 1931 *b*) After administration by stomach or rectal tube, McClure (1943) found lesions in the intervertebral ganglia, chiefly in the lower cervical and lumbosacral groups After ingestion, Burnet and Jackson (1940) recovered virus from the abdominal autonomic nerves, and abdominal lymph glands.

Evidently, therefore, virus entering through the upper part of the alimentary tract passes to the CNS via cranial nerves (see also section on inhalation above), virus absorbed through the intestines probably passes to the cord via sympathetic pathways.

Toomey's experiments Toomey and his associates have very strongly advocated the rôle of the gastro-intestinal tract in the pathogenesis of poliomyelitis (Toomey, 1931–2 *a*, 1932 *a*, *b*, *c*, Toomey and von Oettingen, 1932–3, Toomey, 1932–3, 1933–4 *b*, *d*, 1934 *a*, *c*, 1934–5 *a*, *b*, *c*, *d*, *1935 b*, *1936 b*, *c*, *d*, *e*, *f*, *1937 a*, *d*, *e*, Toomey and Weaver, 1936, 1937, Toomey and Tischer, 1943) The most important findings are as follows

1 A toxic factor which exerts a depressant and lethal effect on animals has been demonstrated in the urine and fec [illegible]
monkeys This "enteric tox [illegible]
intestinal organisms, such a [illegible]

2 Toomey carries out intestinal injection by clamping off a loop of gut and injecting virus "subserosally" Poliomyelitis follows injections of virus alone, but develops more quickly if the virus is combined with enteric toxin Subcutaneous injections of PCB filtrates or vaccines also render young monkeys more susceptible to subserosal injection of virus

3. Injections of PCB filtrate and virus into the motor area or the lumbar cord produce paralysis almost at once, whereas both components are harmless injected separately.

4 Following subserosal injections, the virus is thought to spread to the central nervous system by vagus and sympathetic nerve fibers The first histological changes in the central nervous system occur within 24 hours, and are noted in the spinal ganglia, and later in the anterior and posterior horns, where there is destruction of nerve cells Microglial proliferation takes place almost coincidentally with the nerve cell destruction Later (not before 5 days) well marked perivascular cuffing is present, but there is no meningitis The lumbar area is the most heavily affected, and usually before the other portions of the cord The fact that in the human disease also, the lumbar cord is frequently first involved has led to the suggestion that the route of infection in man is via the gastro-intestinal tract, but Faber (1935) has pointed out that *the lumbar cord is also first affected following nasal* instillation of virus

5. These experiments have been criticized in that the methods are too drastic and cannot simulate the natural route of infection in man (e g, Flexner, 1936). Toomey has countered with the evidence that, without such drastic measures, monkeys deficient in vitamin D are more readily susceptible to injection of virus than normal monkeys. Conversely, addition of vitamin D to the diet serves to protect animals from infection

11. Other Routes

Intraperitoneal. This route is usually combined with an intracerebral injection given at the same time (e.g, Landsteiner and Popper, 1908, Levaditi and Landsteiner, 1909, Flexner and Lewis, 1909 *a*, 1910 *c*, McIntosh and Turnbull, 1913)

The usual incubation period is not over 14 days, but it has been recorded that monkeys injected with virus may not develop symptoms of infection for as long as 6 to 10 months (Genevray and Dodero, 1934). McClure (1941 *b*) found changes in the sensory portions of the vagus ganglia, the intervertebral ganglia, and sometimes the gasserian ganglia

External ear. Monkeys can be infected after instillation of the external ear (Vieuchange, 1936 *a*) As no lesions of the auditory mucosa, tympanum or auditory nerve occur, it is presumed that the virus spreads to the central nervous system by the nerves innervating the external acoustic meatus

Tonsil Monkeys can be infected by dropping virus on to the tonsillar mucosa (Landsteiner, Levaditi, and Danulesco, 1911) Sabin (1938) infected monkeys by direct tonsillar injection, and found that they developed bulbar paralysis

Pulpal inoculation Aisenberg and Grubb (1943, 1945) inoculated the exposed pulps of the anterior teeth, and produced paralytic attack in 1 animal out of 5, the remainder developing nonparalytic symptoms

Injection after transection of the spinal cord Transection of the spinal cord has been carried out prior to injection of virus intracerebrally (Jungeblut and Spring, 1929–30, Toomey, 1933–4 *a*, *c*, Brodie and Elvidge, 1934) Injections have also been given into the cord below the section (Brodie and Elvidge, 1934). Intrasciatic nerve injections have also been used (Toomey, 1933–4 *a*, *c*)

It should be noted that in these experiments there was free circulation of the cerebrospinal fluid between the two cut portions of the cord. Therefore, if the cerebrospinal fluid was concerned in the spread of the virus, this should have been recoverable from the lower portion despite the section

Following intracerebral injection of such monkeys, it was found that typical poliomyelitis developed with the usual incubation period. Virus and histological lesions were present in the cord proximal to the section, but not distally. Virus [illegible]

of the virus by nerve tracts and not by the cerebrospinal fluid or other means Toomey's experiments, however, do not altogether confirm those of Jungeblut and Spring, or Brodie and Elvidge, for he was able to discover both virus and lesions

in the distal portion of the cord after intracerebral injection, and lesions proximally after intrasciatic injection He suggested that the virus had spread to the cord by the sympathetic nerves. Howe and Bodian (1941 *a*, 1942 *a*), like Toomey, found that virus could progress around a section of the cord. After intracerebral or nasal inoculation, virus and lesions were found in the distal portion. After sciatic nerve inoculation, virus might be found in the cord above the transection; they suggested that the paravertebral sympathetic chains were involved. A number of animals was subjected to bilateral sympathectomy of 2–3 ganglia at the level of the transection, virus was then inoculated into the lumbar cord. Virus progressed into the remainder of the CNS, they suggested that it spread from the lumbar cord to the wall of the gut via sympathetic fibers, and thence to the brain stem via the vagus.

At any rate, it would seem that all are agreed that the cerebrospinal fluid plays no part in the dissemination of the virus in this type of experiment.

Testicular injection gives a negative result (Jungeblut and Thompson, 1929–30)

Contact infection. Until recently there were no instances recorded of contact infection in monkeys, and there is every reason to believe that this is an extreme rarity. Howe and Bodian (1944 *b*) isolated virus from the stools of 2 uninoculated chimpanzees quartered for 6 months in cages adjoining those of a rhesus monkey inoculated nasally with a positive stool. "Spontaneous" poliomyelitis of monkeys has not been proved to occur. Any such apparent occurrence would raise the question of whether the monkeys had been used previously or whether they had become infected by contact.

THE FEATURES OF EXPERIMENTAL POLIOMYELITIS IN THE MONKEY

Speaking generally, the features of poliomyelitis are similar whatever the route of infection, any special differences or modifications have already been discussed under the heading of "routes of infection." These features have been described repeatedly, but comparatively little has been added to the early report of Flexner and Lewis (1910 *c*), and the following account is largely based on their paper, as well as personal observations

Incubation Period

The incubation period can vary from 4 to 33 days, but the great majority shows the first signs of nervous involvement after 7 to 10 days Certain monkeys will be found to be more resistant than others, but a batch of animals injected together usually shows the first manifestations more or less concurrently.

Incubation is partly dependent on dosage Small amounts cause infection after prolonged periods, and very large amounts may infect after a shorter period (Brodie, 1933 *b*, Moore *et al*, 1942, Moore and Kessel, 1943) The disease tends to be less severe if the incubation is prolonged.

Nonparalytic Attacks

After inoculation by any route, monkeys and chimpanzees may suffer from nonparalytic attacks, and recover before paralysis sets in (Brodie and Wortis, 1934, Kling *et al.*, 1942 *a*, *b*, Aisenberg and Grubb, 1945, Bodian and Howe, 1945 *a*). Such a diagnosis can be made only by histological examination, or isolation of virus from stool Sabin and Ward (1941 *c*) for example, found destruction of an appreciable number of anterior horn cells, with mesodermal-glial inflammation Bodian and Howe (1945 *b*) studied the condition in chimpanzees, and found that virus might be excreted in the stool for 3 days to 8 weeks after inoculation The changes in the brain and cord were similar to those in paralytic cases The severity of the pathological changes varied from a fully developed distribution of lesions in the brain and cord to mild scattered lesions in the brain only They found no support

for the occurrence of a purely systemic or peripheral form of poliomyelitis. The central nervous system was always involved.

Paralytic Attacks

In the prodromal period, the animal shows a rise in temperature and changes in the CSF (see below). It may be nervous, tremulous, and excited, with erect hairs. Spasticity of muscles may be noted, probably due to lesions in the brain only (*Bodian*, 1946).

The onset is usually sudden, the animal appearing to be more or less healthy, say in the evening, and the next morning showing an established paralysis. This paralysis is seen most commonly in both legs, and next most frequently in either one leg, one side of the face (Fairbrother and Hurst, 1930), or one arm. In certain rare cases where the cerebrum is severely involved, death eventuates in an apoplectic fit, before paralysis can appear.

Course

In severe infections, the paralysis extends rapidly to involve all the limbs. In more moderate cases it may progress rapidly, but only for about 24 hours, from which time slow improvement sets in. The animal is usually to be found sitting quietly in the cage, but on rousing, moves about as actively as the extent of paralysis allows. Teeth grinding and whining noises may be heard. In fatal cases, death occurs usually from 1 to 6 days after the onset of paralysis In convalescence, some degree of muscular wasting and atrophy develop in limbs which were severely paralyzed, in limbs less involved, however, there may be complete recovery, so that the animal is able to move and jump about as well as before. Such recovered animals prove immune on reinoculation A partly recovered monkey may suffer a fatal relapse (see Heaslip, 1938 *a*)

The transitory nature of the paralysis in some monkeys may be explained in two ways (Sabin and Ward, 1941 *c*) If only a few nerve cells are destroyed, normal function can be maintained with a reduced number of cells They found cells showing lesser degrees of damage in such animals, and suggested that probably not all nerve cells attacked by the virus are irreparably damaged

Taylor (1919) carried out an extensive series of blood counts in experimental poliomyelitis, and reported a polynucleosis in the preparalytic stage, with a lymphocyte crisis at the onset of symptoms The preparalytic polynucleosis was confirmed by Harmon, Shaughnessy, and Gordon (1931), but they did not agree that a lymphocyte crisis occurred

The carbohydrate mechanism is said to be deranged in experimental poliomyelitis (Jungeblut and Resnick, 1936). The blood sugar level does not actually show any alteration, but there is an increased tolerance for glucose, as evidenced by a slow response to the glucose tolerance test. This increased glucose tolerance does not depend on the state of involvement of the nervous system, but is evidently a systemic disturbance, possibly of endocrine origin

Normally, gold chloride solution causes a precipitation of brain and cord extracts The serum of monkeys suffering from poliomyelitis can prevent this action, whereas normal serum cannot The property is acquired as early as the disease as the second day, and disappears during convalescence. The stabilizing property of the serum resists 65° C for half an hour (Jungeblut, 1930–1 *a*).

An excellent index of the onset of nervous symptoms in the infected monkey is afforded by study of the temperature chart (Kramer, Hendrie, and Aycock, 1930, Jungeblut, 1931, Harmon, Shaughnessy, and Gordon, 1931, Brodie and Wortis, 1934) The temperature rises, even to 105° F. (normal monkey temperature up to 103° F) during approximately the 3 days before the first clinically recognizable manifestations of infection, with the progress of the disease the temperature drops to subnormal The terminal hypothermia is not associated with a lowered vitamin

in the distal portion of the cord after intracerebral injection, and lesions proximally after intrasciatic injection He suggested that the virus had spread to the cord by the sympathetic nerves. Howe and Bodian (1941 *a*, 1942 *a*), like Toomey, found that virus could progress around a section of the cord After intracerebral or nasal inoculation, virus and lesions were found in the distal portion. After sciatic nerve inoculation, virus might be found in the cord above the transection; they suggested *that the paravertebral sympathetic chains were involved*. A number of animals was subjected to bilateral sympathectomy of 2–3 ganglia at the level of the transection, virus was then inoculated into the lumbar cord. Virus progressed into the remainder of the CNS, they suggested that it spread from the lumbar cord to the wall of the gut via sympathetic fibers, and thence to the brain stem via the vagus.

At any rate, it would seem that all are agreed that the cerebrospinal fluid plays *no part in the dissemination of the virus in this type of experiment*

Testicular injection gives a negative result (Jungeblut and Thompson, 1929–30)

Contact infection. Until recently there were no instances recorded of contact infection in monkeys, and there is every reason to believe that this is an extreme rarity. Howe and Bodian (1944 *b*) isolated virus from the stools of 2 uninoculated chimpanzees quartered for 6 months in cages adjoining those of a rhesus monkey *inoculated nasally with a positive stool*. "Spontaneous" poliomyelitis of monkeys has not been proved to occur. Any such apparent occurrence would raise the question of whether the monkeys had been used previously or whether they had become infected by contact.

THE FEATURES OF EXPERIMENTAL POLIOMYELITIS IN THE MONKEY

Speaking generally, the features of poliomyelitis are similar whatever the route of infection, any special differences or modifications have already been discussed under the heading of "routes of infection." These features have been described repeatedly, but comparatively little has been added to the early report of Flexner and Lewis (1910 *c*), and the following account is largely based on their paper, as well as personal observations.

Incubation Period

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In experiments conducted over a period of about a year, fluids obtained in the early stages showed a predominance of polymorphs, with lymphocytes in excess at the onset of paralysis. Then for some reason later in the year's work there came a period when the cell pictures were chiefly monocytic.

REFRACTORY STATE OF REGENERATING NERVE CELLS

Howe and Bodian (1939 *b*, 1942 *a*) demonstrated this phenomenon in a series of monkeys infected by intranasal inoculation. In one group, the sciatic nerve was sectioned 3–4 days before the development of paralysis in the legs. The anterior horn cells of the sectioned and control sides were both completely destroyed, the destruction of the cells on the sectioned side appeared to begin earliest. In another group, the sciatic nerve section was carried out earlier, between 6 and 57 days before the onset of leg paralysis. In this group, the anterior horn cells on the control side were all destroyed by the action of the virus. On the sectioned side, the vast majority of cells giving rise to the sciatic nerve was spared by the virus.

A similar refractory state of neurons has been demonstrated in the olfactory bulbs (1941 *b*, 1942 *a*). They sectioned the olfactory tracts, and found that after a few days no changes occurred in the bulbs on nasal inoculation. This refractory state was associated with shrinkage and chromatolysis of the mitral cells, and lasted for a considerable period.

In their work on sciatic inoculation fully described on page 974, Howe and Bodian showed that axons will not conduct virus when they are regenerating following section. Evidently, therefore, both neurons and axons undergoing metabolic disturbance associated with regeneration are refractory to attack by virus.

DISTRIBUTION OF VIRUS IN EXPERIMENTAL POLIOMYELITIS

A number of observations has already been mentioned, when discussing the routes of inoculation.

Central nervous system. Brodie (1932 *a*, 1933 *a*) has estimated the virus content of various parts of the central nervous system at the height of the disease. The spinal cord, brain stem, midbrain, and diencephalon contain 92 per cent. of the total virus content, while the basal ganglia, cerebral cortex, and cerebellum, though weighing 3 times as much, only contain 8 per cent.

Virus has not usually been detected in the cord before the onset of paralysis, but is constantly present in large quantity at its height. Thereafter, it rapidly decreases in amount, so that about 3 weeks after the appearance of paralysis the cord may prove noninfective (see, e.g., Fairbrother, 1930, Brodie, 1932–3). The "growth curve" of virus (Lansing strain) in the cord following intracerebral injection has been investigated by Bodian and Cumberland (1947). A period of exponential multiplication occurs over less than 2 days, the average peak being before the onset of paralysis, the average period of maximal concentration is sustained only through the day preceding paralysis and the first day of paralysis. A sharp decline in multiplication takes place on the second day of paralysis, but virus persists in small quantities for at least 2 weeks.

A rare case has been recorded by Brodie (1934–5 *b*) where a monkey showed the presence of virus in the cord without any accompanying histological lesions.

Cerebrospinal fluid. After direct intrathecal injection, the virus is demonstrable for at least 5 days, but is usually absent at the onset of paralysis (Clark and Amoss, 1914, Hurst, 1931).

After other routes of injection, virus may be found, but this is a rare occurrence (Flexner and Lewis, 1910 *c*, *d*, Fairbrother and Hurst, 1930, Hurst 1930, 1932). However, Schaeffer and Muckenfuss (1938) have claimed that virus enters the cerebrospinal fluid rapidly after intracerebral injection.

B_2 reserve (Kolochine-Erber and Raffy, 1944, 1946). In certain cases, the dromedary type of chart may be found, with two well marked rises of temperature in the incubation period (Harmon, Shaughnessy, and Gordon, 1931).

According to Brodie and Wortis (1934), poliomyelitis infection of monkeys may present in 1 of 4 forms (1) nonparalytic (see above), (2) the ordinary spinal form, with or without a prodromal period; (3) a short progressive type like Landry's paralysis, (4) a diphasic type.

Physiological Reactions of Peripheral Nerves

The physiological state of the peripheral nerves after intracerebral injection of virus has been investigated (1, Covell and O'Leary, 1932; 2, Bishop, Heinbecker, and O'Leary, 1932, 3, Heinbecker, Bishop, and O'Leary, 1932; 4, O'Leary, Heinbecker, and Bishop, 1932; 5, Covell, 1932)

The investigation was carried out by using neutral red to demonstrate degenerating myelin, by testing the nerve conduction rates, and by applying galvanic stimulation to the spinal cord and examining for muscle contraction in the area supplied The following is a résumé of the main conclusion of these investigators

(*a*) During the late preparalytic and early paralytic stage there is a definitely increased irritability of the peripheral nerves as indicated by an increased conduction rate and shorter refractory period It should be mentioned that some days before the end of the incubation period there are definite modifications in chronaxia, but that these occur equally in paralytic and nonparalytic cases (Chauchard *et al*, 1934). The nerve fiber shows definite cellular alterations, probably due to an intrinsic action of the virus

(*b*) For a considerable part (4 to 10 days) of the paralytic stage, many nerve fibers can themselves conduct nerve impulses Stimulation of the cord from the first days of paralysis onward fails to elicit a response, however, the anterior horn cells being unable to conduct impulses

(*c*) The nerve cells, though damaged as regards impulse transmission, continue to exert a trophic influence over the nerve fibers

(*d*) In the last stages of paralysis, even stimulation of the nerves fails to elicit a response.

(*e*) Stimulation applied direct to the muscles always elicits contraction

The Cerebrospinal Fluid

Following nasal instillation of virus, changes are always found in the cerebrospinal fluid, even if no symptoms of infection are manifest (Gay and Lucas, 1910, Flexner, 1933 *a*, *b*, 1935 *a*) Even 48 hours after nasal instillation, there develops a definite pleocytosis, the cells being mainly lymphocytes, although in the early stages polymorphs may constitute 60 per cent. of the total count Less constantly, there is an increase in globulin The rise in cell count, which often corresponds with a pyrexial period, may reach 70 cells per c mm. after 48 hours, and 300 cells per c.mm after 4 days In the prodromal period a usual figure is 1,000 cells per c.mm., mainly lymphocytes.

As is well known, monkeys frequently resist one nasal instillation though succumbing on repetition Examination of the cerebrospinal fluid after each such inoculation shows, however, that the characteristic reaction just described has occurred. At the onset of paralysis the majority of cerebrospinal fluids shows a colloidal gold curve with a well-marked luetic reaction, with the progress of the infection this returns to normal (Jungeblut and Khorazo, 1930)

Following intracerebral injection also, changes are to be found in the cerebrospinal fluid, and are of the same nature as those just described, pleocytosis and globulin being found, often at least 48 hours before the onset of paralysis (see Mollaret and Erber, 1934) No change was detected in the vitamin C content of

poliomyelitis (Kasahara and Gammo, 1938)

ed the cytology of fluid removed from the

maximum at the stage of complete paralysis

which lasted for another few days. In such cases the olfactory bulbs showed histological lesions as already described On the other hand, if the bulbs were removed no fever developed

The second method of preventing poliomyelitis following nasal instillation consists in treating the relatively exposed olfactory nerve endings with chemical substances It was shown by Armstrong and Harrison (1935) that the nasal instillation of 4 per cent. sodium alum sulfate secured the survival of almost three-quarters of a batch of monkeys subsequently inoculated intranasally with virus (see also *Haber and Coquoin-Carnot, 1937*)

Later, Armstrong and Harrison (1936 *a*) showed that 3 to 6 instillations of picric acid, with or without alum, were more effective than alum only Also, it was found that the effects were cumulative, 6 instillations giving more protection than 3 Picric acid was also found to be effective (Schultz and Gebhardt, 1936) After such a course, the nasal mucosa is resistant to infection for from 1 to 2 months (Sabin, Olitsky, and Cox, 1936) Armstrong and Harrison also showed that unless the instillation was adjusted to a pH sufficiently acid to cause coagulation of protein, no protection resulted (1936 *b*)

Other substances found to be effective were picric acid plus sodium aluminum sulfate (Armstrong and Harrison, 1936 *b*, Sabin, Olitsky, and Cox, 1936), 3 per cent tannic acid (Sabin, Olitsky, and Cox, 1936, see also Olitsky and Cox, 1934), 1 per cent. nitrophenol, 1 per cent trinitrocresol, and 1 per cent mercurochrome (Schultz and Gebhardt, 1936), pituitrin S and adrephine (Kramer, Grossman, and Parker, 1937, 1938) Later reports suggest that zinc sulfate is the best of all substances so far tested (Schultz and Gebhardt, 1936-7, Olitsky and Sabin, 1937) As described elsewhere, these results have been applied to man, but with negative results

It has been said that not only does nasal instillation of such chemicals protect against infection by the nasal route but also by the intravenous route, thus, Armstrong (1936 *a*) found that poliomyelitis did not develop in monkeys with chemically treated nasal mucosae on injection intravenously This confirms the parallel observation of Lennette and Hudson (1934-5) who injected monkeys intravenously after section of the olfactory tracts, and found that no infection developed However, certain apparently contradictory reports have appeared Thus, German and Trask (1938) reported that bilateral olfactory neurectomy did not inhibit the development of infection following intravenous or intracutaneous inoculation (see also Toomey, 1939 *a*) Further, intranasal zinc sulfate has been said not to prevent the development of infection after intravenous inoculation (Toomey and Takacs, 1938 *a*)

The effect of intranasal instillation of 1½ per cent zinc sulfate is to cause necrosis of the mucous membrane of the olfactory area By the end of 3 to 4 days, however, the epithelium is again normal (Schultz and Gebhardt, 1938)

A large number of chemicals was tested by Schultz and Gebhardt (1942) who found that they differed considerably in their ability to induce resistance Zinc sulfate was the most effective because it caused the severest damage There was an extensive coagulative necrosis of the olfactory epithelium which separated *en masse* from the lamina propria New epithelium grew in later The resistant state might not be established for 2 or 3 days after application Probably animals can always be infected 3 to 6 months later

Chemotherapy

Various drugs have been said to exert some action either in preventing or modifying the attack (Jungeblut, 1930-1 *b*, Hornus and Haber, 1934) antimony, potassium tartrate, neostibosan, mercurochrome, chaulmoogra oil, hexylresorcinol, and solganal Injections of vitamin C have been said to modify the infection (see p 1011)

A number of drugs has been found devoid of effect hexamethylamine, sodium ricinoleate, acriflavine, and tryparsamide (Jungeblut, 1930-1 *a*, Kolmer and Rule, 1933-4 *b*) Luminal, insulin, and metrazol shock did not influence the infection (Jungeblut, 1940) Continuous intravenous hypotonic saline produced no apparent benefit (Kramer, Geer, and Himes, 1942) Potassium chlorate does not appear to have much effect (Arthus *et al*, 1939, Saucier and Stewart, 1940, Levinson and Milzer, 1941).

Nervous ganglia. The virus can be found in the intervertebral, gasserian and abdominal sympathetic ganglia; histologically, there are to be found interstitial changes of perivascular cuffing, and cellular infiltrations with some neuronophagia (Flexner, Clark, and Amoss, *1914 b*; *Hurst, 1929*).

Peripheral nerves. The virus can be found in the sciatic nerve at the height of infection following intracerebral injection (Nicolau, Dimancesco-Nicolau, and Galloway, 1929).

Blood. The virus is very seldom to be found in the blood of infected monkeys (Flexner and Lewis, *1910 c*; *Flexner and Clark, 1911*; *Clark, Fraser, and Amoss,* 1914; Gordon and Lennette, 1939). Melnick (1944 *b*) examined one recently isolated strain that showed a special tendency to invade the blood.

Salivary glands. The virus has rarely been found in this site (Landsteiner and Levaditi, *1909 b*).

Nose and throat. The virus can be demonstrated in the following sites nasal mucosa (Flexner and Lewis, 1910 *c*; Landsteiner, Levaditi, and Danulesco, 1911), tonsil and peritonsillar region (Flexner and Lewis, 1910 *c*, Flexner and Clark, 1911, Landsteiner, Levaditi, and Danulesco, *1911*); *nasopharyngeal mucosa* (Flexner and Lewis, 1910 *b*, *c*, Flexner and Clark, 1911, Landsteiner, Levaditi, and Danulesco, 1911), even after 5½ months (Osgood and Lucas, 1911).

Lymph nodes Virus may be found in the regional lymph nodes following subcutaneous injection (Flexner and Lewis, *1910 a, c*). After intracerebral and intraperitoneal injection, Kling, Olin, and Gard (1938) isolated virus from inguinal and mesenteric glands.

Spleen. Virus has been demonstrated in the spleen (Lennette, 1937).

Intestinal tract. Virus can be recovered from the intestines or stools of monkeys, and particularly of chimpanzees, infected by the usual routes (Kramer, Hoskwith, and Grossman, 1939; Melnick, 1943 *a*, 1944 *b*, Howe and Bodian, 1944 a, Melnick and Horstmann, 1947) Melnick (1946 *b*) examined stools by his ultracentrifugation technique, and found that they might be positive in the incubation period. Virus appeared more readily when the inoculation was given peripherally, subcutaneous and intracutaneous inoculation gave positive stools more commonly than intracerebral inoculation. Several strains acted in this fashion. Trask and Paul (1941 *b*) also found virus in the stool after intracutaneous inoculation A number of workers has recovered virus after infecting animals by the gastro-intestinal tract (see above)

TREATMENT OF EXPERIMENTAL POLIOMYELITIS

Treatment of the Olfactory Nerves

Schultz and Gebhardt (1933–4*b*) were the first to show that nasal instillation does not produce poliomyelitis if the olfactory bulbs and tracts are previously destroyed *This important observation has been confirmed by* Lennette and Hudson (1934–5), Brodie and Elvidge (1934), and Howe and Ecke (1937–8 *b*) In these experiments, the olfactory bulbs and tracts were usually cauterized, and some days or months later, the animals were inoculated intranasally. Control untreated *monkeys* developed poliomyelitis, but those that were treated survived The treated monkeys were not, however, resistant to a subsequent injection given intracerebrally

Lennette and Hudson (1934–5) made the additional observation that poliomyelitis did not develop after intravenous injection in monkeys with severed olfactory tracts, this suggests that any virus entering the blood stream is excreted into the nasal passages, and thence ascends to the brain by the olfactory nerves. As the 5th and 7th nerves are not damaged by these operative procedures, it is evident [illegible] does not travel along them, otherwise poliomyelitis would still develop [illegible] oted that if the olfactory tracts only were severed, [illegible] was actually an attack of *fever after 3 to 7 days*

of the nose or nasopharynx into the cervical sympathetic ganglia, and thence to the upper thoracic cord (see p. 978).

Monkeys can also be infected by pharyngeal swabbing, ingestion, or stomach tube After swabbing or ingestion, virus probably reaches the brain stem by the 5th and perhaps the 9th and 10th nerves. When virus is absorbed from the intestines it is probable that it spreads by the sympathetic fibers to the sympathetic ganglia and cord, or by the vagus to the brain stem (see p 979).

Howe and Bodian (1942 *a*), as a result of their extensive experiments, suggested that virus can reach the CNS by passage along both motor and sensory fibers They did not find the sympathetic fibers to be especially susceptible, although they were sometimes concerned, e g., in spread from the intestine, and spread after transsection of the cord

Following subcutaneous inoculation, virus can be detected in regional lymph nodes, and thereafter generalizes, presumably by the blood (see p. 978).

Dermal inoculation may also prove infective, even apparently in the absence of intact nerve fibers in the area (see p. 978), presumably virus must leave the area by lymph or blood

Blood may become infected with virus not only by direct intravenous injection, but also through infected lymph. It is extremely difficult to infect by the intravenous route unless the blood brain barrier is artificially rendered more permeable (see p. 978) It is possible that virus in the blood may not invade the CNS through the blood brain barrier but by excretion into the nasal passages, and thence via the olfactory nerves

We may conclude that after certain methods of inoculation, virus may spread by blood or lymph to the CNS Even in such cases, however, neural pathways may also be involved

The rôle of the cerebrospinal fluid It has been suggested that virus, having reached the cerebrospinal fluid, either by perineural lymphatics or by the blood, is disseminated by the fluid throughout the central nervous system Little attention need be paid to this theory, because a *sine qua non* of its acceptance would be the frequent demonstration of virus in the cerebrospinal fluid but it is found only after direct intrathecal injection, or after intravenous injection if aseptic meningitis is produced by intraspinal injection of serum (Flexner and Amoss, 1914 *a*). The upholders of this theory postulate that the virus passes into the parenchyma of the cord along the perivascular spaces (see, e g, Harbitz and Scheel, 1907 *a*, *b*, Clark and Amoss, 1914). Only under exceptional circumstances, however, such as do not occur in poliomyelitis, is this the direction of flow in these spaces, normally the flow is from within outward, and not vice versa In confirmation, Kempf and Soule (1939) were unable to recover from the CNS any significant amount of antisheep

tions of Hurst and others (*vide infra*) show, however, that nerve cell destruction is the primary change, and not meningitis Furthermore, any areas of meningitis present do not necessarily overlie the areas of greatest parenchymatous change, as should occur if the virus spreads directly inward It may fairly be assumed, therefore, that the cerebrospinal fluid plays little part in the primary dissemination

The Spread of Virus in the Central Nervous System

The classical work on the spread of virus following intracerebral, intrathecal, and intrasciatic injection using histological methods is that of Hurst and of Fair-

Sulfonamides have proved quite ineffective in preventing the onset or modifying the course of infection (Kelson, 1937; McKinley, Acree, and Meck, 1938, Toomey and Takacs, 1938 *b*, 1939, Rhodes and van Rooyen, 1938; van Rooyen and Morgan, 1943).

Blood obtained from an infected *M. mulatta* and treated with UVL did not modify the severity of the disease (Toomey and Takacs, 1943).

Here it may be mentioned that Jungeblut and Kopeloff (1931) found that a preexistent infection with *T. equiperdum* resulted in a prolonged incubation

Physical Treatment

X-rays produced harmful effects (Amoss, Taylor, and Witherbee, 1919, Lenz and Jungeblut, 1931–2).

Pyrotherapy applied in the incubation period was not capable of preventing the subsequent development of poliomyelitis, according to Jungeblut and Kopeloff (1931). However, Wolf (1934–5) reported that he was able to prevent the disease by treating during the incubation period.

PATHOGENESIS OF EXPERIMENTAL POLIOMYELITIS

In this section will be discussed the method whereby the virus spreads from the site of entry to the central nervous system, many facts in this connection have already been mentioned in this chapter.

A number of reviews on this subject, with reference to the human disease also, has appeared in recent years (e g, Report of the International Committee, 1932, Faber, 1933, Lovett, 1934, Doerr and Seidenberg, 1936–7; Kauders, 1937 *a*), Faber's (1933) paper puts forward convincingly the theory of spread by axonal routes

Portal of Entry to the Body

Monkeys can be infected most readily by intracerebral or intranasal inoculation, by exposure to atomized virus, or by application of virus to the sciatic nerve, other routes of infection are the intra-ocular, subcutaneous, dermal, intravenous, intraperitoneal, intratonsillar, and gastrointestinal

Passage of the Virus to the Nervous System

When virus is introduced other than by direct inoculation in the brain or cord, there are various paths open for spread to the CNS The simplest method of tracing the spread of virus is by histological examination, especially of the various ganglia, where the involved neurons occur and show degenerative changes. It appears that care must be taken, however, in drawing conclusions from the mere presence of lesions in ganglia Bodian and Howe (1947) reported that "control" chimpanzees may show accumulations of inflammatory cells similar to those found in inoculated animals.

By nerve paths Numerous observations have shown that following inoculation by peripheral routes, virus travels to the central nervous system by nerve paths For example, following inoculation of the sciatic nerve, virus spreads centrally, and the rate has been measured Virus can only spread in fibers that are both anatomically and physiologically normal Regenerating fibers will not conduct. Virus spreads actually in the axon, and not in the sheath

Following intra-ocular injection, virus probably travels via the optic nerve or by the ciliary ganglia and oculomotor nerves (see p 976)

Following intranasal inoculation, virus spreads by the olfactory nerves to the bulbs, and thence by the tracts to the brain This spread can be prevented by the application of chemicals that destroy the nerve endings, or by section of the fibers (see p. 986).

When animals are exposed by inhalation of atomized sprays, virus passes to the CNS either by the olfactory nerves, or by the afferent fibers of the trigeminal to the gasserian ganglion. Less commonly the pathway consists of the sympathetic fibers

of the nose or nasopharynx into the cervical sympathetic ganglia, and thence to the upper thoracic cord (see p 978).

Monkeys can also be infected by pharyngeal swabbing, ingestion, or stomach tube. After swabbing or ingestion, virus probably reaches the brain stem by the 5th and perhaps the 9th and 10th nerves When virus is absorbed from the intestines it is probable that it spreads by the sympathetic fibers to the sympathetic ganglia and cord, or by the vagus to the brain stem (see p. 979)

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We may conclude that after certain methods of inoculation, virus may spread by blood or lymph to the CNS Even in such cases, however, neural pathways may also be involved

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As a preliminary to the actual entrance of the virus to the cord a stage of meningitis is postulated (e g, by Flexner and Amoss, 1914 *b*) The experimental observations of Hurst and others (*vide infra*) show, however, that nerve cell destruction is the primary change, and not meningitis Furthermore, any areas of meningitis present do not necessarily overlie the areas of greatest parenchymatous change, as should occur if the virus spreads directly inward It may fairly be assumed, therefore, that the cerebrospinal fluid plays little part in the primary dissemination of the virus following injection by routes other than intrathecal In all probability, however, once the virus has actually reached the parenchyma of the central nervous system, it may be spread secondarily by the cerebrospinal fluid, accounting for the patchy meningitis usually present (Fairbrother and Hurst, 1930)

The Spread of Virus in the Central Nervous System

The classical work on the spread of virus following intracerebral, intrathecal, and intrasciatic injection using histological methods is that of Hurst and of Fair-

brother. These papers have already been mentioned in the appropriate sections (Hurst, 1929, 1930, 1932; Fairbrother and Hurst, 1930). Numerous other workers have also studied the problem by histological methods (Faber and Gebhardt, 1932–3; Pette, Demme, and Kornyey, 1932, Hudson, Lennette, and Gordon, 1936, Howe and Ecke, 1937–8 *a*, Luhan, 1937, Bodian and Howe, 1939; Howe and Bodian, 1942 *a*).

Brodie (1932 *a*, 1933 *a*) investigated the question by means of titrations of various parts of the nervous system, and his observations confirmed those of the histological workers.

The general opinion of these workers is that virus spreads from the olfactory tracts or site of cerebral inoculation to many parts of the brain, by definite, often decussating, nerve paths In the cerebral cortex, however, the nerve cells are relatively resistant to the virus and little intrinsic change takes place therein, although the interstitial tissues react. Correspondingly, only a small quantity of virus can be demonstrated in the cerebral cortex Virus also spreads via the basal nuclei and medulla to the cord, where it can be demonstrated in large quantities. The results of transection experiments (see p. 981) have helped to confirm the validity of the theory of axonal spread. If virus is injected intracerebrally in a monkey in which the spinal cord has been severed, then only the proximal portion contains virus or shows lesions.

Howe and Bodian (1942 *a*) have shown that in the preparalytic stage, after the onset of fever, there may be a localization of histological changes along certain paths, depending on the portal of entry. By the time paralysis develops, virus has reached the preferential paths from the portal of entry, e.g, the nose or cortex, to the cord, and has passed rostrally and caudally along these paths There then follows a spread of virus to less susceptible centers. This secondary spread may occur even in the early stages when virus is inoculated in the frontal lobe. The final picture is similar with any portal of entry. Howe and Bodian (1942 *a*) point out that the final distribution of the histological changes is determined by at least 3 factors the differential susceptibility of certain centers, accessibility of the center to virus as determined by neuronal connections, the portal of entry

THE PRIMARY TISSUE REACTION IN EXPERIMENTAL POLIOMYELITIS

Poliomyelitis virus causes degenerative changes in the neurons and a mesodermal-glial inflammatory reaction. The work of Hurst (1929) and Fairbrother and Hurst (1930) conclusively showed that the primary attack of the virus is on the neuron

of Bodian and Howe (1941 *a*) and Howe and Bodian (1942 *a*). These authors performed hemidecortication in rhesus monkeys, which caused large areas of the thalamus to become devoid of nerve cells, neuroglia, vessels, and perivascular tissue were left intact Normally the thalamus is susceptible to virus, but when inoculation was carried out onto this neuron-denuded area, the mesodermal-glial inflammatory reaction did not develop. Both focal and perivascular lesions occurred in nearby areas where the nerve cells had not undergone retrograde degeneration.

For some reason, which is obscure, poliomyelitis virus shows a special tendency to attack anterior horn cells, and to a lesser extent neurons in the cerebrum and midbrain. Certain areas are normally highly resistant and show no lesions Even if virus is inoculated directly into such areas, e g, the lateral geniculate body or occipital lobe, mesodermal-glial lesions develop only in the immediate vicinity of the inoculum (Bodian and Howe, 1941 *a*, Howe and Bodian, 1942 *a*)

It seems probable that the attack of virus may be on the cytoplasm rather than the nucleus Bodian (1945) examined a rhesus monkey killed in the acute stage,

neurons in the brain, cord, and spinal ganglia.
ntaining 2-6 nuclei, showed degenerative
always showed identical changes, suggesting
toplasm, and that the nuclear changes are
secondary.

It is quite definitely established that neuronal damage is primary, and not secondary, to nearby perivascular infiltration or overlying meningitis. Howe and Bodian, with other workers, conclude that the mesodermal-glial response is sec-... to the interaction of virus with susceptible neurons, and is not due to a ... tissues

virus is not only neurotropic
(Hurst), but is neuronotropic,
its growth upon, living nerve
cells of certain types.

THE PATHOLOGICAL FEATURES OF EXPERIMENTAL POLIOMYELITIS

The Nonparalytic Stage

From experiments on chimpanzees and rhesus monkeys it has been concluded that the disease can be spontaneously arrested at any stage after entry of the virus into the CNS, before or even after the onset of paralysis (Bodian and Howe, 1941 *d*, Howe and Bodian, 1942 *a*)

The minimal degree of change observed in nonparalytic cases is a regional destruction of neurons and mesodermal-glial infiltration, at and near the site of entry of virus into the CNS. The cord may not be involved, and the full distribution of cerebral changes may not occur.

After intranasal inoculation, there may be a severe encephalitis rostral to the medulla, but no involvement of the cord

The maximal degree of pathological change observed in nonparalytic cases is practically equivalent in distribution and severity to the pathological involvement in paralytic cases The absence of detectable paralysis is due to the fact that the distribution of the destroyed neurons is too scattered to involve a single functional muscle group sufficient to produce clinically evident paralysis.

The Paralytic Stage

These features have, of course, been recorded by many authors (e g, Flexner ... 1911 *a*, Hurst, 1929, 1931, Pette,
following description, referring mainly to the ... intracerebral injection,[1] is largely that given by Hurst in his paper (1929), now regarded as one of the most important contributions to the histological study of poliomyelitis

The cord Irrespective of the portal of entry, the cord shows the greatest intensity of inflammatory change of any part of the CNS The changes predominantly involve the lumbar and cervical enlargements, although no portion is entirely free The lumbar cord is said to be particularly involved after intestinal and intrasciatic injection

... variable feature, but is usually
...phocytes predominate in the in-
... numbers of polymorphs may

[1] For a fuller description of the changes in the early stages of poliomyelitis following intracerebral injection, see the section on "routes of infection" In this section also, the pathological features of poliomyelitis following other routes of injection are more fully described

be present. The areas of meningitis are not necessarily related to underlying lesions in the gray matter. After intrasciatic injection, lumbar meningitis is usually intense

Perivascular infiltration is also a variable feature; the infiltrating cells are mainly lymphocytes, but in the early stages polymorphs may also be present (see Fig. 43). It is important to note that perivascular infiltration may be quite absent in the presence of severe nerve cell destruction, and therefore is not the primary change

Diffuse and focal cellular infiltrates are frequently present, especially in the anterior and posterior horns, and gray commissure, the cells are usually lymphocytes, although in the severer cases polymorphs may be found

Definite proliferation of the microglia occurs, and using Del Rio Hortega's silver stain, Hurst was able to show the true nature of these cells; they had puzzled many earlier workers who called them "polyblasts" or "elongated glial cells" The



FIG 43 Photomicrograph of an histological section through the spinal cord of a rhesus monkey infected with poliomyelitis virus, showing degeneration of nerve cells together with lymphocytic infiltration around two blood vessels Stained hematoxylin and eosin × 250

microglia may undergo degenerative changes consisting of thickening and eventual disappearance of the processes, and coarsening and vacuolation of the protoplasm

The most important change of all, however, is that in the nerve cells In the anterior horns of the cervical and lumbar regions, up to 50 per cent. of the available cells may be destroyed (see Fig 43) Cells in the posterior horns (especially Clarke's column) may undergo similar changes In the preparalytic stage the nerve cells show at most only early degenerative changes, but within 24 hours they have become totally destroyed Increasingly severe lesions can usually be observed with the progress of the infection, e g, central chromatolysis, complete dissolution of the Nissl substance, swelling and vacuolation of the cytoplasm, the nucleus disappears or alternatively becomes hyperchromic and excentric, neuronophagia ensues and polymorphs and microglia remove the effete cell In certain very acute cases the nerve cells appear to be killed more or less instantaneously and show little in the way of change. The nucleolus may be enlarged or mulberry-shaped (Hurst,

1931). Parallel changes to those detected by ordinary staining methods have been studied by the micro-incineration technique.

If the animal recovers after a severe attack of paralysis, there is a noticeable diminution in the numbers of anterior horn cells, with reactionary increase in the microglia and neuroglia

Burnet, Jackson, and Robertson (1939 *b*) found the changes in cynomolgus monkeys to be more diffuse than those in rhesus animals. The inflammatory reaction was intense in the cynomolgus monkey, and characterized by the presence of

CSF were associated
nfiltration.
ges (pial and perivascular infiltrations) are related to the 4th ventricle. There is some nerve cell destruction, but neuronophagia is rare Lesions around the 4th ventricle are most pronounced after intrathecal injection (Hurst, 1932).

Midbrain and basal ganglia These areas may show little else than focal and marked perivascular infiltrations

Cortex and cerebellum. Meningitis and focal and perivascular infiltration occur somewhat irregularly, nerve cell degeneration is only common in the motor area Usually, therefore, the changes in these areas are slight, but Brodie (1934 *d*) has recorded an autopsy on a monkey injected intracerebrally where the cerebral cortex and parts of the cerebellum showed a considerably severer reaction than usual. Burnet, Jackson, and Robertson (1939 *b*) also found changes in the motor area irrespective of the route of inoculation.

Spinal ganglia. Changes have been described in the neurons by Hosselet and Erber (1937), the chondriome becomes granular and eventually disappears, while the Nissl bodies degenerate.

Inclusion bodies have been found by certain authors, both in experimental and

to 4 μ in diameter, and may be surrounded by an unstained halo, as many as 10 may be present in any one nucleus, they do not give the Feulgen reaction and are not doubly refractile to polarized light. It is probable that these inclusions represent nucleolar extrusions

Micro-incineration studies These studies have been carried out by Patton (1933–4, 1934) The spinal cords of monkeys injected intracerebrally were removed at varying periods of clinical involvement Some sections were stained by the usual methods, and others were micro-incinerated (see Ch X). The large anterior horn cells were carefully examined, and with the progress of the infection were seen to undergo 3 definite changes

(*a*) *Edema* Ordinary HE sections showed chromatolysis, the nucleus, at first swollen, later became shrunken and excentric, and the chromatin showed peripheral margination Intranuclear inclusions varying up to 3 to 4 μ in diameter were found, sometimes as numerous as 10 per cell Micro-incineration of similar cells showed a progressive diminution in the amount of mineral ash The inclusions yielded a small quantity of ash

(*b*) *Increased mineral ash* No longer did the nucleus and cytoplasm show approximately parallel changes, for in this stage one might be considerably more degenerated than the other HE sections showed the cell to have become vacuolated and hyperchromic, with a granular cytoplasm and contracted nucleus, neuronophagia might be present

Micro-incinerated cells showed a most striking increase in the cytoplasmic ash, corresponding to the granularity found in the HE sections When neuronophagia occurred, the nerve cells

(*c*) *Nec* f ash
present in The
cell was m

The Neuromuscular Mechanism

Carey (1943, 1944) examined the neuromuscular mechanism. The earliest changes were hyperemia and perivascular infiltration of the intramuscular blood vessels. This hyperemia was noted in weakened but nonparalyzed muscles, prior to alteration in motor end plates. On the first day of paralysis, some end plates were retracted and some hypertrophied and granular. About 20 per cent. of the motor plates were absent in paralyzed muscle on the first day, and within 2–4 days 50 per cent. had disappeared

From 1–4 days after the onset of paralysis, masses of axonic substance, stained with gold, were found in close relationship to the degenerating tree of innervation The degeneration appeared to begin in the motor end plates, and then extended in a centripetal direction through the epilemmal axons. Carey advanced the hypothesis that *the changes observed in the neuromuscular mechanism in the early stages are* the result of abnormal excitation of the secretory mechanism of the motor end-plates resulting in exhaustion of the gold-staining axonic substance, leading to denervation at the myoneural junction.

The Chronic Stage

There have been few reports of histological examinations in monkeys which have survived a typical attack of poliomyelitis, Warburg (1931) has, however, contributed an important paper on the subject, reviewing previous work but more particularly reporting personal observations. The most important finding was that inflammatory changes might still be present, indicating that the infection was active despite apparent recovery. Degeneration of nerve cells and perivascular infiltrations were the evidences of infection, while microgliosis and astrocytosis indicated repair.



CHAPTER LXXXV

INFECTION OF ANIMALS OTHER THAN THE MONKEY

COTTON RATS

THE MANAGEMENT of cotton rat colonies presents some difficulties and has been

Certain strains of virus have been adapted to this animal, and most work has been done with the Lansing strain adapted to the eastern cotton rat, *Sigmodon hispidus hispidus* by Armstrong (1939 *a, b*, 1941, Lillie and Armstrong, 1940 *a, b*). The infected animal develops roughened fur, jumps when agitated, shows flaccid paralysis, and dies within 2–4 days of the appearance of symptoms. It is most susceptible to intracerebral injection, but according to Weaver (1944, 1945, 1946 *a, b*), it can also be infected by various peripheral routes. Infection *in utero* or by milk does not occur (Weaver and Steiner, 1944).

Histologically, the changes are those of polioencephalitis, similar in characteristics and distribution to those seen in man and monkey (Lillie and Armstrong, 1940 *a, b*) The earliest lesions occur in the cord, the severest reaction is in the medulla and pons; well-marked lesions also occur in the cerebellar roof nuclei and midbrain The cerebral cortex is relatively little involved The nerve cells show necrosis, and there is polymorph infiltration and neuronophagia, there is also focal and diffuse cellular gliosis, swelling of the vascular endothelium, and perivascular

insing strain also infects *S*

partial inanition, influenced

5, 1946 *a, b*). Female cotton rats, or rats deficient in vitamin A, were especially susceptible to paralysis when virus was given by the intratonsillar or other peripheral route (Weaver, 1946 *a, b*).

The Flexner MV and the Phila strains can produce paralysis in cotton rats (Toomey and Takacs, 1940 *c*, 1941 *a, b, c*, Toomey, Frohring, and Takacs, 1943).

The SK strain was adapted to cotton rats after isolation in monkeys, and at first caused an inapparent infection (Jungeblut and Sanders, 1940 *a*).

MICE

Work with Monkey-Pathogenic Strains

Many workers failed to adapt monke
Lewis, 1910 *a, c*, Kolmer and Rule, 1
Harrison, 1934–5, Vieuchange, 1936 *b*, I
1940 *b*, Paraschivesco and Papazolu, 194
and Hudson, 1943) A number of other
seemed suggestive, but in view of the typical changes now known to be produced by murine strains, it is doubtful whether they actually succeeded in adapting virus to the mouse (Nungester, 1932–3; Weil, 1932–3; Brodie, Goldberg, and Stanley, 1935, Toomey and Phelps, 1935–6).

The following strains of virus can be propagated in mice Lansing, MEF1, MM, SK, Y-SK, Ph, and Theiler's virus According to the strain, the appearances are

[1] References are appended at the conclusion of Ch. LXXXVIII, p 1026 et seq.

The Neuromuscular Mechanism

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The Chronic Stage

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...on was active
...r infiltrations
...icated repair.



deficient animals surviving infection may become paralyzed when given adequate thiamine, after a prolonged incubation (Rasmussen *et al.*, 1944).

A similar, but less marked, increased resistance is noted in mice subjected to restriction in calories only (Foster *et al.*, 1943, 1944 *a*, *b*, Rasmussen *et al.*, 1944).

As regards riboflavin, mice deficient in this factor show a slightly greater resistance than controls to the Lansing strain (Rasmussen, Waisman, and Lichstein, 1944).

Deficiencies in pyridoxine, inositol, and biotin do not appear to influence susceptibility to Lansing or Theiler strains (Lichstein *et al.*, 1945)

Mice fed a diet deficient only in calcium pantothenate show a definitely increased resistance to Theiler's virus, but practically none to the Lansing strain (Lichstein *et al*, 1944).

Endocrines. Infected mice treated with thiouracil invariably succumb earlier than controls, whereas those treated with thyroid extract show incubation periods longer than controls (Holtman, 1946 *b*).

As regards estrogens, mice treated with alpha-estradiol dipropionate or diethylstilbestrol show an increased resistance to the mouse-hamster virus inoculated nasally, but not when it is injected peritoneally (Foley and Aycock, 1945), they could not demonstrate increased resistance to Lansing virus inoculated cerebrally.

Temperature. Mice acclimatized to a temperature of 13° C. were found to develop symptoms on intracerebral injection after 11 days, whereas with animals acclimatized to 32° C the symptoms came on in 5 days (Holtman, 1946 *a*).

In apparent contradiction, it was found that the incubation period of the disease in young mice inoculated intracerebrally with the SK strain in standard dose was considerably lengthened in the summer months (Jungeblut, Sanders, and Feiner, 1942).

Chemotherapy in Mice

A number of sulfonamides and sulfones has been tested in infection of mice with the Lansing strain and found to be ineffective (Coggeshall and Maier, 1942; Kolmer and Rule, 1942, Levaditi, 1942 *b*). Kramer, Geer, and Szobel (1944) found 190 compounds useless in modifying the course of infection with the Lansing strain Potassium chlorate had no effect in mice infected with the Lansing strain (Levaditi, 1942 *b*) Anesthetization with ether after infection was also ineffective (Sulkin, Zarafonetis, and Goth, 1946)

About the only promising results in chemotherapeutic experiments are those of Wood and Rusoff (1945). Using the MM strain injected intraperitoneally, they found that intraperitoneal injections of trypan red, brilliant vital red, and Congo red lowered the incidence of infection (Congo red also lowered the incidence of infection in cotton rats)

Neoarsphenamine had a slight effect in murine infections (SK and other strains), the effect was antagonized by cysteine and glutathione, fuadin and sulfapyridine enhanced the effect very slightly (McKinstry and Reading, 1945)

GUINEA-PIGS

The majority of workers has found these animals insusceptible to monkey-pathogenic strains (Flexner and Lewis, 1910 *a*, *c*, Stimson, 1918, Kolmer and Rule, 1933–4 *a*, Brodie, 1934–5 *c*, Woolpert and Harrison, 1934–5, Kolmer, Rule, and Werner, 1937, Yen and Hsu, 1941, Schwab, Woolpert, and Hudson, 1943) Chor (1933) found that injection in splenectomized animals gave general lymphoid hyperplasia, fever, and death within 2 weeks, the lesions were not suggestive of poliomyelitis Guinea-pig fetuses could not be infected (Schwab, Woolpert, and Hudson, 1943)

After 70 transfers in mice, the SK strain developed an increased pathogenicity for guinea-pigs (Jungeblut and Sanders, 1941) A fixed strain of cavian virus was

those of encephalitis or flaccid paralysis, the incubation period also varies with the strain.

Features of Infection with Murine Strains

The Lansing strain has been particularly studied in mice (Lillie and Armstrong, 1940 *b*, Levaditi, 1942 *b*; Smith, 1943 *a*, Vieuchange, 1945, 1946). Animals are usually infected intracerebrally, but may develop infection also by the intraperitoneal route Levaditi could not infect mice intranasally, intradermally, by the sciatic nerve, or by the mouth The incubation period is from 2–92 days, but is usually 4–14 days The animals develop a fatal paralysis, but some may die without paralysis. If passage material is mixed with autolyzed mouse brain, the incubation period is said to be considerably reduced (Milzer and Byrd, 1947).

The histological appearances are similar in character and distribution to those seen in cotton rats infected with the same strain (see p. 995), monkeys, or human beings (Stefanopoulo, Duvolon, and Étévé, 1942, Ehrich and Foster, 1944, Foster and Ehrich, 1944, Vieuchange, 1945, 1946).

The content of adenosine triphosphate is greatly increased in the brain, phosphocreatine and residual organic phosphate are markedly decreased (Kabat, 1944)

The distribution of virus after intracerebral injection has been studied Virus can be found in the blood and spleen for 1–2 hours after inoculation (Smith, 1943 *b*). Mice which develop paralysis within 5 days after intracerebral inoculation show as much virus in the brain as in the cord and medulla, after 5 days little virus is isolated from the brain, parts of the cord and medulla of mice becoming paralyzed up to 4 weeks after inoculation contain as much virus as do specimens obtained in the first 5 days (Herrarte and Pearson, 1943).

Half an hour after intraperitoneal injection, virus can be recovered from the blood and spleen, it persists in the blood for 7 and the spleen for 12 hours (Smith, 1943 *b*).

The SK strain has also been studied in mice. When adapting the strain from cotton rats, the first few passages caused an inapparent infection (Jungeblut and Sanders, 1940 *a*).

After over 200 serial intracerebral passages, this strain increased in virulence from a titer of 1/1 million to not less than 1/1 billion (Jungeblut, Sanders, and Feiner, 1942) Bourdillon and Moore (1942) found the infectivity of a standard intracerebral inoculum of 0 03 c.c to be from 10^{-7} to 10^{-8}.

Young mice are more susceptible to peripheral inoculation than older animals, but intracerebral injection is equally fatal in young and old animals (Jungeblut, Sanders, and Feiner, 1942).

The SK strain is present in the brain, cord, blood, spleen, liver, and adrenals of infected mice (Jungeblut and Sanders, 1940 *a*)

The SK strain also attacks the anterior horn cells, and with an increase in virulence there is an increased affinity for the cerebral tissues (Wolf, 1942)

The *MM strain* has similar properties to the SK, and kills mice within a few days

Influence of Various Factors on the Susceptibility of Mice

Strain of mouse Cross-bred mice (Swiss white mother, wild gray father) have been found more susceptible to the Lansing strain than pure-bred white mice (Holtman, 1947).

Nutrition. Thiamine-deficient mice have been shown to be more resistant than usual, particularly as regards the incidence of paralysis, to the Lansing or Theiler viruses, vice versa, the incidence of paralysis is greater in mice on a high thiamine diet than in those on a low diet (Foster *et al*, 1942, Rasmussen *et al*, 1944, Waisman *et al.*, 1945). This resistance is only partly explicable by the accumulation of pyruvate or other metabolites (Waisman *et al*, 1945). In some instances, thiamine-

and Rule, 1933–4 *a*, Brodie, 1934–5 *c*; Woolpert and Harrison, 1934–5; Swan, 1942), the bush rat, *Cricetomys gambianus* (Findlay, Anderson, and Haggie, 1946), *Microtus*, *Neotoma*, *Peromyscus*, *Perognathus*, *Dipodymus* (Kessel and Stimpert, 1940); horses, calves, goats, pigs, sheep, dogs, and cats (Flexner and Lewis, 1910 *a*, *c*), pigeons and chickens (Kolmer, Rule, and Werner, 1937, Schwab, Woolpert, and Hudson, 1943); hedgehogs and agoutis (Grossman and Kramer, 1937–8) Fetuses of white mice and white rats could not be infected (Schwab, Woolpert, and Hudson, 1943).

The Flexner MV strain adapted to cotton rats did not infect white rats even when suffering from vitamin B_1 deficiency (Toomey, Frohring, and Takacs, 1943).

Chicks injected intracerebrally with the rodent-adapted MM strain harbored virus and developed antibodies, but there was no definite adaptation (Brutsaert, Jungeblut, and Knox, 1946 *a*).

Jaculus jaculus and *Arvicanthis barbarus* were not susceptible to Lansing virus (Durand, 1945).

The Lansing strain did not infect ground squirrels *Citellus tridecem lineatus* and *C. richardsonii*

obtained by passage in guinea-pigs (Jungeblut, Feiner, and Sanders, 1942) The animals develop flaccid paralysis and show typical lesions of the anterior horn cells They are susceptible to murine SK virus by intracerebral, intravenous, intraperitoneal, and subcutaneous inoculation, but the cavian strain produces paralysis only on intracerebral or intravenous inoculation. This cavian passage virus produced only mild lesions in the monkey.

The SK strain shows a characteristic attack on the anterior horn cells and the features resemble those of the disease in man (Jungeblut and Sanders, 1941; Wolf, 1942).

HAMSTERS

The Chinese hamster was not found to be susceptible to a monkey pathogenic strain (Yen and Hsu, 1941). Young hamsters are susceptible to the M-hamster virus inoculated intraperitoneally, and develop paralysis within 5 days (Dalldorf and Whitney, 1943).

The Lansing strain could be passed intracerebrally in the Syrian hamster, and a fatal paralysis developed, a few survivors showed muscular atrophy and contracture The pathological features were typical of poliomyelitis (Plotz, Reagan, and Hamilton, 1942). Durand (1945) also found the Syrian hamster susceptible after an incubation of 4–7 days, fatal paralysis developed, and conjunctivitis might be present.

OTHER SUSCEPTIBLE RODENTS

The Lansing strain infects a number of other rodents *Microtus* (Kessel and Stimpert, 1940), *Neotoma* (Kessel and Stimpert, 1940), *Peromyscus* (Kessel and Stimpert, 1940, Gordon, 1945 *a*), *Ondatra zibethcus* (muskrats) (Gordon, 1945 *a*).

Adult specimens of *Meriones shawi* develop symptoms in 3–13 days after intracerebral inoculation of the Lansing strain, paralysis often occurring (Durand, 1945). In very young animals, subcutaneous, intraperitoneal, or nasal inoculation proves positive Durand found the following to be less regularly susceptible *Psammomys obesus, Gerbillus hirtipes, G atoni, Dipodillus campestris (quadrimaculatus), D dodsoni*

OTHER SUSCEPTIBLE ANIMALS

Frauchiger and Hoffmann (1938) claimed to have produced paralysis and muscular atrophy in a cow inoculated with human material.

Ferrets were found to be insusceptible by Kolmer, Rule, and Werner (1937), but Patŏcka (1941) found one particular strain that produced degenerative changes in the anterior horn cells.

RABBITS

Certain workers have claimed that monkey-pathogenic strains can be transmitted (Dahm, 1909. Krause and Meinicke, 1909, 1910, Levaditi and Landsteiner, 1910 *a*, Ceccaldi, 1943) Other workers have not been able to demonstrate any specific evidence of infection with such strains (Flexner and Lewis, 1910 *a*, *c*, Stimson, 1918, Kolmer and Rule, 1933–4 *a*, Brodie, 1934–5 *c*, Kolmer, Rule, and Werner, 1937, Toomey and Takacs, 1940 *a*, Yen and Hsu, 1941, Kempf, Pierce, and Soule, 1941, Schwab, Woolpert, and Hudson, 1943, Toomey, Pilcher, and Rossman, 1943) Rabbit fetuses could not be infected (Schwab, Woolpert, and Hudson, 1943)

The murine SK virus is not pathogenic to rabbits (Jungeblut and Sanders, 1940 *a*).

INSUSCEPTIBLE RODENTS AND OTHER ANIMALS

The following animals have been found insusceptible to monkey-pathogenic strains rats and jerboa mice (Flexner and Lewis, 1910 *a*, *c*, Stimson, 1918, Kolmer

(c) In 1933, Gildemeister reported that he had secured growth of the virus in tissue culture, the medium, consisting of Tyrode's solution, monkey serum, and chick embryo brains, was incubated at 37° C. in Carrel flasks Poliomyelitis developed in monkeys inoculated with the 8th subculture. Kast and Kolmer (1937) were not, however, able to confirm

(d) Using human embryos (3 to 4 months old) obtained by cesarean section and cultured in Tyrode's solution, Sabin and Olitsky (1936 b) also were able to obtain growth of the virus Typical infection was produced in monkeys by 1 c c. of the 6th subculture which represented a dilution of the original inoculum of 1:1,000,000

(e) More recently the SK strain has been cultivated. Jungeblut and Sanders (1940 a) obtained growth in embryonic mouse brain in ox serum ultrafiltrate. In further studies they found that the optimum pH was 7 3-7 6 (Sanders and Jungeblut, 1942) On prolonged subculture, the virus showed a loss of infectivity for mice on peripheral inoculation, although the potency by the intracerebral route remained constant. The SK strain can be titrated *in vitro* in embryonic mouse brain (Huang, 1943).

CULTIVATION IN EGGS

Monkey-pathogenic strains have not been cultivated on the chorio-allantois (Burnet, 1935, Kast and Kolmer, 1937). Gard (1943 c) has claimed that the virus may propagate in young embryos and invade the brain.

The MM rodent-adapted strain could not be propagated by the allantoic route (Brutsaert, Jungeblut, and Knox, 1946 a). The SK and Lansing strain have been propagated by the chorio-allantoic and yolk sac routes and invade viscera (Schultz and Enright, 1946).

REACTION TO PHYSICAL AGENTS

The influence of pH on the infectivity of the Lansing strain in mice has been investigated by Hammon and Izumi (1941) who found that a definite quantity of virus can be rendered several times more infective at pH 4 than 7. Faber and Dong (1946) found this strain was completely inactivated at pH 1 0 in 5 minutes, at pH 2.0 partial inactivation began at 2 hours and was complete at 4 hours. At pH 3 0 and 4 0 there was no inactivation at 4 hours The virus is electronegatively charged (Olitsky, Rhoads, and Long, 1929) Bourdillon (1943-4 a) investigated the electrophoretic mobility of the unpurified SK strain X-rays have no definite effect on the virus (Lenz and Jungeblut, 1931-2) Monkey and mouse strains are inactivated rapidly by ultraviolet light, probably in less than 1 second (Toomey, 1937 b, Jungeblut, 1937-8, Levaditi, 1942 d, Milzer, Oppenheimer, and Levinson, 1944, 1945) Sunlight, especially in the presence of eosin, also has a destructive effect (Flexner, 1916, Amoss, 1928)

Methylene blue solutions (1 50,000 to 1 100,000) inactivate the virus when exposed to light (Rosenbloom, Hoskwith, and Kramer, 1937-8)

Supersonic vibrations completely destroy the virus (Kasahara *et al*, 1938), material so treated is antigenic (Keeser, 1939) Homogenization of infected tissue increases its activity (Scherp and Chambers, 1936-7)

Numerous observations have been made on the effect of heat on the virus (see Shaughnessy, Harmon, and Gordon, 1930, for bibliography) The general opinion of these workers is that 55° C destroys in up to half an hour Lawson and Melnick (1947) have carried out experiments with rodent-pathogenic strains, using infected nerve tissue, infectivity was reduced by 95 per cent in 30 minutes at 45° C., but over 60° C was required for complete inactivation Milk exerted a protective effect on virus in nerve tissue, virus withstanding 5-10° C. extra The virus survives for at least 50 days at +4° C (Flexner and Lewis, 1910 c)

After desiccation *in vacuo*, the virus probably survives for at least a month (Landsteiner and Levaditi, 1909 b, Romer and Joseph, 1910 b). It retains its virulence

CHAPTER LXXXVI

SOME OTHER PROPERTIES OF POLIOMYELITIS VIRUSES

MORPHOLOGY

COLES (1939)[1] claimed to have identified elementary body-like structures, but they are too small to be seen with ordinary microscopic equipment

Inclusion bodies have been described in the nuclei of infected cells in man and monkey, these have been subjected to micro-incineration, the Feulgen test, and [illegible] they represent nucleolar extrusions [illegible] been taken by Jungeblut and Bour-[illegible]asuring 25–30 mμ in purified preparations (see also Loring, 1947). In unpurified preparations of tissue cultures long thread-like structures up to 5000 mμ were seen. Loring, Marton, and Schwerdt (1946) examined purified preparations of the Lansing strain, and found spherical or slightly asymmetrical particles of 12–34 mμ, no threads were seen

Rod-shaped particles have been seen by Gard (1943 *a*, *b*) and Melnick (1944 *a*) in stools and nervous tissue. Gard, in his studies on the purification of Theiler's virus (see p. 1004), demonstrated rod-shaped particles in purified preparations, structures about 15 mμ by 115 mμ were seen also in material from human cords, and in the stools of healthy as well as infected humans

FILTRATION AND CENTRIFUGATION

Monkey-pathogenic strains have been found to pass through the following filters Chamberland L5, Berkefeld V and N, and with difficulty through W (Flexner and Lewis, 1909 *c*, 1910 *c*, Landsteiner and Levaditi, 1909 *a*, *b*, Amoss, 1928), Seitz filters can also be used.

Monkey-pathogenic strains measure 8–17 mμ by ultrafiltration (Krueger and Schultz, 1928–9, Clifton, Schultz, and Gebhardt, 1931, Theiler and Bauer, 1934, Elford, Galloway, and Perdrau, 1935, Levaditi *et al*, 1936).

The SK strain measures 10–15 mμ (Sanders and Jungeblut, 1942) Levaditi (1942 *c*) obtained a larger figure for the Lansing strain, but probably for technical reasons

Monkey-pathogenic virus can be sedimented in the ultracentrifuge (Schultz and Raffel, 1937–8). The SK strain showed a sedimentation constant around 100 S, with a molecular weight of about 10 million (Bourdillon and Moore, 1942, Bourdillon, 1943–4 *a*). The Lansing strain was found to be sedimented after 4 hours at *18,000 r p m (Silverberg, 1945)*

TISSUE CULTIVATION

Numerous attempts have been made to cultivate monkey-pathogenic strains

(*a*) Early work by Levaditi (1913 *a*, *b*), using the method of Marinesco and Minea (1912), showed that virus in infected spinal ganglia of monkeys conserved its virulence after several passages in monkey plasma

(*b*) Later, work was carried out by Eberson in which suggestive evidence of growth was obtained (Eberson, 1931–2, 1932, 1936, Eberson and Mossmann, 1932–3). The medium (VB), which probably contained living cells, consisted of sheep brain in veal broth After some days turbidity developed, and small elementary bodies measuring up to 0 2 μ in diameter were found Infection could be transmitted to monkeys even after a dozen subcultures (representing a virus dilution of 2×10^{-27}).

[1] References are appended at the conclusion of Ch LXXXVIII p 1026 et seq

PURIFICATION AND NATURE OF THE VIRUS

A number of earlier workers achieved some success in their attempts to purify and concentrate poliomyelitis virus in monkey tissue. The methods used included filtration (Clark, Ainsworth, and Kindschi, 1933–4), adsorption to, and elution from, alumina gel, and concentration *in vacuo* (Sabin, 1930–1, 1932), adsorption to, and elution from, aluminum hydroxide (Schaeffer and Brebner, 1933), half-saturation with ammonium sulfate (Clark, Schindler, and Roberts, 1930), precipitation with safranine (Howitt, 1930–1) Brown and Kolmer (1937–8) attempted unsuc-

orkers has succeeded
weight, probably of
and mouse-infected
order. Most workers

have started with nervous tissue and made use of ether, protein precipitants, and differential centrifugation

Method of Clark, Rasmussen, and White (1941)

Clark, Rasmussen, and White (1941), using ether-extracted monkey tissue, confirmed earlier observations that half-saturation with ammonium sulfate brings down almost all the virus, especially at pH 4 5. The virus appeared to come down with the euglobulin and pseudoglobulin fractions. Virus was further concentrated from the sediment of the purified suspension by repeated ultracentrifugation and distillation *in vacuo*

Method of Bourdillon and Moore

Bourdillon and Moore (1942) used the SK strain The method of concentration in-

dillon (1943–4 *a*, *b*) reported on various constants and on the rate of thermal inactivation of purified material.

Method of Racker (1942)

Racker worked with infected mouse brain tissue The method involved ether treatment, adjustment of pH to 4 0, centrifugation, precipitation with ammonium sulfate, and dialysis After treatment with acetic acid, a crystalline material was obtained, highly infective for mice He did not claim that this material was the virus, as the infectivity factor might merely have been adsorbed thereto

Method of Herrarte and Francis

These workers (1943) purified the Lansing strain by adding protein precipitants to suspensions of brain and cord of mice Using ether and centrifugation, they found nearly all the virus was present in a solid layer under the ether. The virus could be readily dissolved in alkaline buffer to yield a clear material

Method of Loring and Schwerdt

ey found that the purified form of the MVA virus

In 1946, Loring and Schwerdt obtained a material of high molecular weight from the nervous tissue of cotton rats infected with the Lansing strain Etherization and differential centrifugation were employed. The yield after a third cycle of centrifugation was 0 1 to

The same procedures
only 0 02–0 01 mg The
as strongly in favor of
the virus particles to

after desiccation over phosphorus pentoxide for at least 24 days (Landsteiner, Levaditi, and Pastia, 1911 *a*). Melnick (1946 *c*) found that 2 mouse-adapted strains and Theiler's virus maintained their original infectivity for 12 months in the frozen state at − 70° C or − 20° C

REACTION TO CHEMICAL AGENTS

Poliomyelitis virus is glycerol-resistant (e g, Flexner and Lewis, 1909 *c*, Landsteiner and Levaditi, 1909 *a*, *b*, 1910; Flexner, Clark, and Amoss, 1914 *b*, Flexner and Amoss, 1917 *c*, Rhoads, 1929) In 33 to 50 per cent. glycerol it can survive with comparatively little loss of virulence for at least 6 to 8 years (Flexner and Amoss, 1917 *c*; Rhoads, 1929) Moore and Kessel (1943) found that each 7–9 months of storage tended to reduce the titer to the next lower tenfold dilution, but the *severity of the illness was not affected It is relatively ether-resistant* (see, e g, Sulkin and Zarafonetis, 1947).

A large number of compounds has been found to destroy or inhibit monkey strains *in vitro*. citrate (Rhoads, 1931 *a*), potassium permanganate, hydrogen peroxide, menthol, salol, boric acid (Levaditi and Landsteiner, 1910 *d*, *e*), strong urea solution (Mackay and Schroeder, 1936–7); formalin vapor (Romer and Joseph, *1910 b*), *isamine blue, to a slight extent (Hornus and Haber, 1934), chrysoidin* Y, Congo red 4 B, copper sulfate, hexylresorcinol, mercuric chloride, mercurochrome, Unna's polychrome methylene blue, oxyquinoline sulfate, potassium hydroxide, and potassium permanganate (Schultz and Robinson, 1942). Synthetic ascorbic acid was found to render virus noninfective (Jungeblut, 1939) The virus is inactivated by adsorption to cholesterol (Toomey, 1937 *e*), ergosterol, and vitamin D (Toomey and Takacs, 1938 *c*). The virus is relatively resistant to salicylate (Cooke and Best, 1941), and cetamium (Toomey and Takacs, 1945)

The chemotherapy of monkeys is discussed on page 987 and of mice on page 997

REACTION TO BIOLOGICAL AGENTS

Normal testicular extracts produce a marked loss of virulence when mixed with virus *in vitro* (Jungeblut, 1932).

The virus is not destroyed by bile (Landsteiner, Levaditi, and Pastia, 1911 *a*, Levaditi, Kling, and Lépine, 1931) or trypsin (Faber and Dong, 1946) It can survive the gastro-intestinal secretions for at least 2 hours *(Flexner, Clark, and* Dochez, 1912), however this probably depends on pH The virus inactivating agent (VIA) present in normal human nasal secretion, acting powerfully on influenza virus, *has a slight action on the MV strain (Burnet, Lush, and Jackson, 1939)* Allen and Clark (1941) also studied VIA in man, in the naturally resistant calf, they did not find that an extract of the olfactory mucosa neutralized virus

The virus can survive in butter for at least 3 months at −2° C (Kling, *Levaditi*, and Lépine, 1931), in milk for at least 31 days at room temperature (Landsteiner, Levaditi, and Pastia, 1911 *a*), in water for about 3 months at room temperature (Landsteiner, Levaditi, and Pastia, 1911 *a*, Kling, Levaditi, and Lépine, 1929)

ANAEROBIC GLYCOLYSIS OF INFECTED BRAIN

Brodie and Wortis (1934) found no great alteration from the normal when investigating the respiratory mechanism of excised brain and cord from an infected monkey A number of workers has investigated the conditions underlying the *metabolism and anaerobic glycolysis of mouse brains* infected with the Lansing and similar strains (Racker and Kabat, 1942, Kabat *et al.*, 1943, Nickle and Kabat, 1944, Utter, Reiner, and Wood, 1945, Wood, Rusoff, and Reiner, 1945) Purified preparations are said to inhibit glycolysis when added to homogenates of normal mouse brain (Racker and Krimsky, 1946)

CHAPTER LXXXVII

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IMMUNITY IN POLIOMYELITIS. ACADEMIC AND EXPERIMENTAL ASPECTS

ANTIGEN-ANTIBODY REACTIONS

THE ANTIBODY most readily demonstrable in poliomyelitis is the virus neutralizing antibody, although certain other antibody effects can be shown.

VIRUS NEUTRALIZATION

Virus neutralization tests in monkeys.

Until recently, poliomyelitis antibody could be demonstrated only by its ability to neutralize the infectivity of virus on inoculation in monkeys. The expense involved in obtaining a satisfactory result seriously restricted the usefulness of this type of test, which can now be replaced by titration in mice (see below)

Tests in monkeys should be quantitative. For example, 1000 m c p. doses of virus can be tested with varying dilutions of serum (Madsen and Jensen, 1936,[1] Eagles, Jensen, and Henningsen, 1937). Kolmer and Rule (1935 *a*) recommended a constant amount of serum with varying quantities of virus

In the majority of observations, monkey passage virus has been used as antigen, but it would appear preferable to use a local recently isolated strain, in view of

ody may

the test

nonkeys.

Burnet, Jackson, and Robertson (1939 *b*) have recommended the use of cynomolgus animals, using intracerebral or intra-ocular injections, rhesus monkeys can also be injected intra-ocularly (1939 *a*).

Work of Schaeffer and Muckenfuss.

Schaeffer and Muckenfuss (1940) have published a valuable monograph on the neutralization test in monkeys. From their review of the literature, they conclude that the technique used in the past has not conduced to accurate results Many of the published results, especially when a single monkey has been used per sample, have been of little or no value The test is unsuitable for quantitative studies, such as detecting antigenic differences between strains, but is of value in qualitative studies such as epidemiological surveys.

In their own experimental work, designed to improve the test, and reduce the chances of inaccuracy, they used the following basic technique

As a source of virus, they employed the pooled mixture of about 50 monkeys' spinal cords removed at the height of paralysis (MV strain). The cords were then minced mechanically, and placed in a sterile flask with 50 per cent glycerin, and shaken for 12 hours The material was stored at −20° C till required A 5 per cent. suspension of pooled cords was prepared, centrifuged at 2,000 r p m for 15 minutes, and the supernatant (with or without filtration) used as virus

Equal portions of virus and serum (neat or diluted) were placed in test tubes, mixed, and transferred to a fresh set of tubes, held at 37° C for one hour, placed in the refrigerator overnight, and then incubated at 37° C for another hour the next morning The volume of incubated mixture generally inoculated was 1 c c. A 1 c c tuberculin syringe was used, and a ¼" 26 gage needle The inoculation was made 1 cm to the right of the

[1] References are appended at the conclusion of Ch. LXXXVIII, p 1026 et seq

Method of Melnick

Melnick (1943 *a*) concentrated infected stool material by differential ultracentrifugation and etherization He isolated a macromolecular fraction from human, monkey, and chimpanzee stools. Later (1946 *a*, 1947) he introduced the Sharples instrument, he has used a special technique for isolating virus from sewage (see p 963).

Work of Gard

Gard (1943 *a*) has carried out extensive studies on the purification of Theiler's and human strains, using the method of concentration essentially the same as that described on page 931 (see also 1943 *b*). He isolated from mouse brains infected with Theiler's virus a substance identical with the virus protein. The substance was present in all active specimens adequately concentrated, but absent in concentrates from normal mouse brains The preparations, representing 1 5 × 10^{-4} per cent. of the original material, retained 30 per cent. of the total virus content. The infectivity of the purified substance was thus about 200,000 times that of infected brain The infectivity of different preparations varied proportionally with their content of virus protein The purest preparations were homogeneous in the ultracentrifuge, and had a sedimentation constant equal to that of the virus The size (i e., breadth) of the virus rods was found to be 10–15 mμ by ultrafiltration The molecules were rod-shaped or filamentous, the width being found to be 12–13 mμ by sedimentation or diffusion data, or 15 mμ by the electron microscope Statistical analysis of titration experiments suggested that most of the virus particles appeared in larger aggregates which probably dissociated on dilution Sedimentation and diffusion experiments disclosed a certain inhomogeneity of the presumed virus protein, indicating that the particles in reality were aggregates of smaller units end to end This was confirmed in electron micrographs, where the length of the elementary particles was estimated at about 115 mμ Most fibers had a length of about 1 μ and corresponded to 8–9 particles The molecular weight of the protein was calculated to be about 57,000,000, referring to aggregates of about 5 elementary particles

Small amounts of a substance resembling the material considered to be pure mouse virus were recovered from human brain and cord This was not obtained in a pure state, but electron micrographs showed structures of the same size and shape as the mouse virus

Further work was carried out with stools From human stools 3 main components were isolated, one of which resembled closely the substance obtained from infected nervous tissue, and held to be pure virus A pure preparation of this stool component presumably retained all the original infectivity However the stools of healthy persons yielded a similar substance

The feces of normal mice yielded a component of the same type The component from young animals proved infective, but not that from older animals Similar noninfective components were isolated from the feces of pigs and wild brown rats

Intestinal protein could not be distinguished from virus protein by sedimentation data or electron micrographs It was composed of molecules of the same size and shape as those of the virus In infective specimens, the viral activity was concentrated along with this protein

The diffusion constant (D_{20}) of the substance derived from infected mouse brains was 0 32 × 10^{-7}, while that of the intestinal components of man, white mouse, and brown rat was 0 11 × 10^{-7} From the diffusion and sedimentation constants, assuming cylindrical and nonhydrated molecules, the following figures were calculated nervous material—molecular weight 57 × 10^6, particle size 12 5 × 580 mw, intestinal material, m w 200 × 10^6, particle size 12 2 × 2150 mμ

A pure preparation of intestinal material of human origin was found to be probably a nucleoprotein

The intestinal proteins were antigenically active in rabbits, and those from man, white mouse, and brown rat were related

It is suggested tentatively that intestinal protein is an avirulent or non-neurotropic variant of poliomyelitis virus It is a normal inhabitant of the intestines, is absorbed and deposited in lymph nodes with the consequent acquisition of the immune state The intestinal protein (virus) is nonpathogenic, but under the influence of exogenous factors, e g, weaning in the mouse, or climatic factors in man, many undergo variation to a neurotropic form

Complement Fixation

Kolmer and Rule (1935 *b*) concluded that fixation may occur between antiserum and monkey virus, but Harrison (1939) and Raffel and Schultz (1940) could find no evidence of this phenomenon.

Precipitation

[illegible]

Allergy

It was found that if virus was injected in the spleen of monkeys immune to poliomyelitis (either by recovery from infection or by vaccination), sudden death occurred (Brebner, 1931–2) Death did not result unless about 7 weeks had elapsed [illegible] lytic attack, That such [illegible] c) reaction was sug- [illegible] mucosae, necrosis of the center of the liver lobules, and hemorrhages in the medulla.

Monkeys which have been injected parenterally on a number of occasions develop a prolonged febrile disturbance 3 to 4 days after intracerebral injection Jungeblut (1931) has suggested that this altered response is indicative of an allergic phenomenon

Miscellaneous Reactions

It has been suggested that colloidal gold solution is precipitated by salt in the presence of poliomyelitis antibody (Eberson, 1930–1; Eberson and Mossmann, 1931–2). Schultz *et al* (1934), however, were unable to distinguish between convalescent and normal sera, the precipitation was nonspecific and appeared to be closely related to the absorption of foodstuffs from the bowel

Some experiments have been performed on the agglutination of sensitized bacteria (Hallauer and Renz, 1945, Roberts, 1945).

ACTIVE IMMUNITY INDUCED BY LIVE VIRUS IN ANIMALS

In Monkeys and Chimpanzees

On recovery from a paralytic attack induced, for example, by intracerebral or intranasal injection, monkeys usually show a marked resistance to a challenge injection of virus, even if given intracerebrally (Flexner and Lewis, 1910 *a*, *c*, Levaditi and Landsteiner, 1910 *a*, Leiner and Wiesner, 1910 *a*, *b*, *c*; Amoss, 1928, Kessel and Stimpert, 1941) The state of resistance probably develops from 7–10 days after the onset of paralysis

In general, the severer the attack, the more marked the subsequent resistant state (Toomey, 1936 *c*, 1938 *a*, 1939 *b*, Moore *et al*, 1942, Moore and Kessel, 1943)

Resistance is more likely to be overcome if the challenge injection is of an heterologous strain, massive doses can also overcome the resistant state (Toomey, 1938 *a*, Kessel and Stimpert, 1941, Howe and Bodian, 1942 *a*)

Challenged animals not completely resistant may develop localized paralysis or nonparalytic attacks

Chimpanzees developing a subclinical attack and excreting virus following dermal inoculation or ingestion of virus resisted a challenge dose of the homologous strain given by the same route (Melnick and Horstmann, 1947).

Toomey and Weaver (1939) reported that monkeys recovered from poliomye-

the coronal suture, and the contents inoculated in the
Melnick (1943 *a*) concentr- ... esia. Young rhesus monkeys of 6–8 lbs weight were
and etherization. He is- ... t 30 days, daily temperatures were taken, all animals
panzee stools La-
special tech- ... d in determining the source of inaccuracies and
... l observations
... ate titer of pooled human *convalescent serum*
... inoculations were performed in triplicate
... e remained healthy not infrequently developed
... pparently neutralized behaved like diluted virus,
... of small amounts of virus from the mixtures
... ntration of serum or virus had any appreciable effect
... that irregularities were less frequent when serum and
... s, when incubation was carried out overnight, and when
... ere inoculated (e g , 0 25 c c).
... showed that in most cases an intracerebral inoculum rapidly
... y with larger inocula and longer needles For example, in one
... onkeys injected with an apparently neutral mixture intracerebrally
th. ... failed to develop poliomyelitis, while 4/10 animals injected with
the s ... hrough ⅞" needles contracted the disease
6 It ... that animals were less likely to die of poliomyelitis on inoculation of
neutral m... s in the subarachnoid space over the hemispheres, or in the cisterna magna

Neutralization tests in mice.

The discovery of human strains of virus transmissible to mice has allowed of titration of antibody in these animals As adequate numbers can be used, more accurate results can be obtained Armstrong (1941) found agreement between titrations in mice and monkeys Mice may be injected intracerebrally with virus-serum mixtures, using the Lansing strain, 8–12 mice per dilution are used, and they should be observed for 21 days (Armstrong, 1941, Harford and Bronfenbrenner, 1941, Hammon and Izumi, 1942 *a*, *b*, Young and Merrell, 1943, Armstrong and Davis, 1945, Brown and Francis, 1945, Olitsky and Findlay, 1946, Morgan, 1947 *a*). The MEF1 strain can also be used (Olitsky and Findlay, 1946).

Neutralization tests in cotton rats.

Cotton rats can be used for the titration of antisera by the intraperitoneal or intracerebral injection of mixtures of serum and the Lansing strain (Hallauer and Renz, 1945).

Effect of antiserum on infectivity of virus.

Poliomyelitis antiserum neutralizes but does not destroy virus *in vitro* Complement is not required for the reaction (Fairbrother, 1930). Active virus can be recovered from neutral mixtures by various procedures Virus has been recovered by cataphoresis (Olitsky, Rhoads, and Long, 1929) Dilution with saline renders neutral mixtures infective (Schultz, Gebhardt, and Bullock, 1930–1, 1931) Schaeffer and Muckenfuss (1938, 1940) found that when long needles were used for the intracerebral injection of apparently neutral mixtures infection resulted, but with short needles the mixture proved noninfective. Neutral mixtures act as antigens in monkeys and stimulate the production of antibodies (Romer and Joseph, 1910 *a*, *b*, Brodie, 1932 *b*, Kramer and Schaeffer, 1933–4, Kramer, Schaeffer, and Park, 1934)

Virus neutralizing properties of tissues.

Normal monkey brain or cord added to virus *in vitro* exerts no neutralizing effect (Jungeblut, 1932) The cords of convalescent monkeys either show no neutralizing action, or at most an irregular one (Landsteiner, Levaditi, and Pastia, 1911 *a*, Jungeblut, 1932).

In Rodents

Guinea-pigs recovered from paralytic or symptomless attacks, induced by the SK strain, are resistant to challenge with the homologous virus (Jungeblut, Feiner, and Sanders, 1942) Cotton rats could be rendered resistant to an intracerebral challenge following the intra-ocular, intratonsillar, intracardiac, intraperitoneal, or subcutaneous administration of virus, administration by other routes was less effective (Weaver, 1946 *a*).

Mice can be rendered resistant to an intracerebral challenge of the Lansing strain by intraperitoneal or subcutaneous injection (Levaditi, 1942 *a*, Kramer and Geer, 1945, Milzer and Byrd, 1947). This refractory state lasts for about 85 days (Levaditi, 1942 *e*)

Sanders and Jungeblut (1942) rendered mice relatively resistant to a mouse-passed strain of the SK virus by the inoculation of tissue cultured virus by peripheral routes. Preparation of mice with estrogen decreased the numbers showing active disease, and increased the numbers becoming immune by subclinical attack (Aycock and Curley, 1947).

ACTIVE IMMUNITY INDUCED IN ANIMALS BY INACTIVATED OR ATTENUATED VACCINES

In Monkeys

Physically or chemically treated virus Attempts have been made to modify the virus so that it retains immunizing properties while losing pathogenicity. Flexner (1935 *b*) concluded that no evidence had been advanced to show that such a result could be obtained.

Virus attenuated by heat at 45° C. for half an hour induced some resistance (Romer, 1912) Desiccated virus has also been employed with some success (Levaditi and Landsteiner, 1910 *b*) Virus inactivated by supersonic vibration has been used by Kasahara (see Paul, 1947 *a*).

Tannin-treated virus failed to immunize monkeys regularly (Olitsky and Cox, 1936). Chloroform-treated virus was also ineffective (Kolmer and Rule, 1934 *a*).

Phenolized vaccines have proved effective, but probably only in preparations where the virus is attenuated and not destroyed (Kraus, 1911, Aycock and Kagan, 1927, Cowie 1934–5, Brodie, 1935 *b*)

Most attention has been paid to formalin and sodium ricinoleate·

Brodie's vaccine It was shown that monkeys could be immunized, so as to withstand even intracerebral injection, by a course of formolized cord given subcutaneously (Erber and Pettit, 1932)

Brodie also found that formalin-treated virus was antigenic (1934 *a*, 1934–5 *a*) Virus was treated with 0 1 per cent. formalin for 16 to 48 hours, and used to immunize children (see p. 1015) Later, Brodie recommended virus inactivated with 0 05 per cent formalin (1935 *a*)

Monkeys can readily be immunized by an injection of 5 c c. of 10 per cent. infective cord treated with formalin, becoming as resistant as monkeys recovered from an attack of poliomyelitis, and developing serum antibodies. Unlike the immunity resulting from injections of active virus, that following formolized virus is not proportionate to the dose used, being optimal after injection of 0 5 gm. of cord (Brodie, 1935 *b*) As regards the activity of Brodie's vaccine, Brodie and Park (1935 *b*) found that 5 c c of virus inactivated by 0.1 per cent. formaldehyde for 8 to 12 hours at 37° C was noninfective Trask and Paul (1936), however, found that 0 1 per cent. formaline did not destroy the virus in 24 hours in the refrigerator.

Kramer (1936 *a*) attempted to immunize monkeys with a vaccine prepared by Brodie's method, even after large loses, antibodies were only rarely developed, and animals succumbed to test injections of 0 01 c c of virus Similar results were obtained by Olitsky and Cox (1936) Thus, at the present, it can hardly be accepted

litis with residual signs again became paralyzed when inoculated with bacterial cultures, the cords did not show changes characteristic of poliomyelitis

The work of Howe and Bodian has shown that whether a rhesus monkey develops a second attack on challenge depends partly on the strain of virus used, and partly on the degree to which the virus disseminated in the CNS on the first exposure. Only limited portions of the CNS may be refractory (Howe and Bodian, 1941 *e, f*; 1942 *a*).

They found that 2 rhesus monkeys convalescent from an intracutaneous and an intracerebral inoculation, respectively, suffered further paralysis after nasal inoculation of an heterologous strain. A third animal convalescent from an intranasal inoculation showed an extension of lesions after the intranasal and intraocular inoculation of an heterologous virus.

Two "spinal" animals, in which the attack of poliomyelitis was limited to an isolated segment of the cord, contracted typical paralysis in the previously uninvaded portions of the CNS following intranasal inoculation of an homologous virus

Four out of 6 convalescent monkeys showed an extension of lesions, without clinical signs, when the homologous virus was inoculated by a new route.

Animals convalescent from a unilateral intranasal inoculation showed evidence of a fresh invasion in the opposite olfactory bulb, but no extension of paralysis, following a second inoculation of homologous virus in the appropriate nostril.

Two animals developed second attacks when a second inoculation of heterologous virus was given, the intranasal route being used on each occasion

Continuing these observations, Bodian and Howe (1945 *b*) found that a chimpanzee recovered from an intracerebral injection sustained a second infection limited to the olfactory bulbs when given virus nasally two weeks later. Another chimpanzee contracted a nonparalytic attack after administration of virus by a stomach tube. When infected orally 9 months later, the animal developed a paralysis of the facial nerve only.

Resistance can also be induced by *injections of live virus*, preferably repeated, without the development of obvious clinical manifestations Refined products have not been found to be significantly more antigenic than cruder preparations (Gordon, 1932–6, Gordon, Harrison, and Hudson, 1935, Kramer, Grossman, and Hoskwith, 1936). Thus virus can be given by the following routes

(*a*) Subcutaneously (Levaditi and Landsteiner, 1910 *c*; Flexner and Lewis, 1910 *c*, Stewart and Rhoads, 1929, Pettit, Erber, and Kolochine, 1932).

(*b*) Intracutaneously (Aycock and Kagan, 1927, Stewart and Rhoads, 1929, Jungeblut and Hazen, 1930–1 *a*, Rhoads, 1930, Brodie, 1932 *b*, Thompson and McKinley, 1934–5); Stimpert and Kessel (1940) found that the immunizing power of different strains varied, and was not related to virulence

(*c*) Intraperitoneally, but this is not very effective (Jungeblut and Hazen, 1930–1 *a*)

(*d*) Intrapleurally (Jungeblut and Hazen, 1930–1 *a*)

(*e*) Application to naso- and oro-pharynx Jungeblut and Hazen (1930–1 *b*) failed to induce resistance by spraying the nose and throat, but others have been more successful (Brodie and Park, 1935 *a*, Kramer, Grossman, and Parker, 1937, 1938, Faber, Silverberg, and Dong, 1943 *a, b*)

(*f*) The intravenous route is not effective Lennette and Hudson (1939) inoculated a number of rhesus monkeys intravenously, and found that practically all succumbed to an intracerebral challenge.

Morgan, Howe, and Bodian (1947) were unable to induce resistance when live monkey virus was given subcutaneously However, they obtained more satisfactory results with large doses of Lansing virus given intramuscularly, antibodies developed, and also resistance to cerebral challenge, the intravenous and subcutaneous routes were not effective.

Bronfenbrenner, 1941). The latter workers titrated antibody by inoculation in mice with Lansing virus as antigen.

Antibodies develop more quickly after injection of live virus by the peripheral routes mentioned in Ch LXXXIV. Antibodies develop after one injection (Rhoads, 1930; Kessel and Stimpert, 1938) Brodie (1934 *b*) found that after a single intracutaneous injection, antibodies appeared after the 6th day, reached their height by the 20th, and lasted for 1 year. However, 2nd injections give a characteristic secondary response, and for the maximum result should be given 10–20 days after the primary stimulus (Brodie, 1934 *b*).

It has been suggested that after subcutaneous injection, certain monkeys and chimpanzees produce antisera potent enough to be used therapeutically (Pettit, Erber, and Kolochine, 1932).

Antibodies develop in monkeys vaccinated with Kolmer's and Brodie's vaccines (see above), phenolized virus, or aluminum-gel virus preparations (Gordon, Hudson, and Harrison, 1939).

In Rodents

Antibodies to the rodent-paralyzing strains develop in cotton rats, mice, and guinea-pigs on recovery from infection. Brown and Francis (1945) found that mice paralyized by an intracerebral injection of the Lansing strain developed neutralizing antibodies 3–6 days after inoculation, whereas the serum of animals becoming paralyzed after 7–9 days had little neutralizing capacity The sera of mice killed 3–6 days after injection, where no paralysis developed, showed as much neutralizing power as those of paralyzed animals killed at the same time.

Antibodies develop in mice vaccinated with ultraviolet irradiated preparations (Milzer, Oppenheimer, and Levinson, 1944, 1945)

In Insusceptible Animals

There is no evidence that the sera of naturally resistant animals neutralize virus (Paraschivesco, 1940, Gordon, 1945 *b*).

Hyperimmune sera can be produced in insusceptible animals.

Horses The fact that only limited quantities of human convalescent serum can ever be available, stimulated attempts to produce antiserum from the horse. Some workers failed to find neutralizing bodies even after repeated injections (e g, Flexner and Lewis, 1910 *e*, Leake, 1918), others were, however, more successful Thus, Neustaedter and Banzhaf (1917) injected a horse with virus-containing filtrates ("digested" with trypsin) and obtained definite virus neutralizing bodies in the serum This work was continued and eventually a highly potent antiserum was produced, neutralizing virus in a dilution of 1 500, whereas human convalescent serum neutralized only in a 1 20 dilution (Weyer, Park, and Banzhaf, 1929, 1931; Rhoads, 1931 *b*; Weyer, 1931–2)

Since that date, numbers of authors have produced immune, even hyperimmune, sera, by injecting horses, often with various purified products (Fairbrother, 1930, Fairbrother and Morgan, 1930, Schultz and Gebhardt, 1930–1 *a*, *c*, 1933–4 *a*, 1934, Schaeffer, 1933–4, Howitt, 1934, Toomey, 1937 *c*, 1938 *b*, 1943 *a*). Such sera may neutralize virus in considerable doses (e g, 1 c c. neutralized 25,000 minimum infecting doses) injected intranasally or intracerebrally. The antibodies are concentrated in the globulin fraction (Toomey, McKhann, and Fahey, 1943).

Pettit's serum A similar serum was produced from the sheep by Pettit (1918), and others have confirmed his original observation (e g, Stewart and Haselbauer, 1928, Howitt *et al*, 1931, Howitt, 1932, 1933, Gordon, Harrison and Hudson, 1934–5, Toomey, 1934–5 *e*, Gordon, Hudson, Harrison, 1939) Pettit also produced a serum from the horse (see Pettit, 1931)

either that Brodie's vaccine is free from active virus, or that it can be used effectively to immunize monkeys

Kolmer's vaccine. McKinley and Larson (1926–7) first showed that sodium ricinoleate might be a useful agent for carrying out immunization of monkeys Kolmer and Rule (1934 *a*, *b*) were able to confirm this observation by finding that subcutaneous and intracutaneous injections of ricinoleated virus conferred slight immunity. The virus was prepared from a 4 per cent. suspension of cord in 1 per cent. sodium ricinoleate, incubated at 37° C. for 24 hours and then refrigerated at 4° to 6° C. for 14 days with occasional shaking. Later, Kolmer and Rule (1936) stated that almost all monkeys injected with 5 doses of ricinoleated vaccine (0 1 to 0 5 c.c per kg.) developed serum antibodies, and the majority was resistant to an intracerebral test injection of active virus

These results were confirmed and amplified when it was stated that for ricinoleated virus to produce effective immunity, sufficient living virus must be present in 0 3 c c. of vaccine to produce paralysis of monkeys on intracerebral injection (Kolmer and Rule, 1937). They further noted that intradermal injection was both safer and more efficient than the subcutaneous route Approximately 1 per cent of monkeys developed paralysis after subcutaneous injection of virus.

Others, repeating Kolmer's work, have failed to immunize monkeys with any regularity, although agreeing with Kolmer that ricinoleated vaccine contains active virus (Olitsky and Cox, 1936, Levaditi, Kling, and Haber, 1936, Kramer and Grossman, 1936).

Virus-serum mixtures Although certain workers have failed to find neutral virus-serum mixtures antigenic (e g., Flexner and Lewis, 1910 *f*), others have found that a more or less effective immunity can be obtained (Romer and Joseph, 1910 *a*, *b*, Rhoads, 1931 *a*, Brodie, 1932 *b*, Kramer and Schaeffer, 1933–4, Kramer, 1936 *b*) It may also be noted that immunity can be produced by simultaneous injection of virus and serum Thus, virus injected intradermally stimulates immunity without producing infection, if it is "covered" by a subcutaneous injection of antiserum given at the same time, or 3 days before or after (Brodie and Goldbloom, 1931, Brodie, 1932 *b*, Flexner, 1932).

Nonspecific injections Monkeys immunized with diphtheria toxoid have been found to survive subsequent injections of poliomyelitis virus (Armstrong and Harrison, 1933), the sera neutralized the virus (Jungeblut, 1934, 1936 *a*)

In Rodents

It has been found that mice can be rendered resistant to the Lansing strain by the intraperitoneal or subcutaneous injection of inactivated formolized virus (Kramer and Geer, 1945)

Levaditi (1942 *e*) found that virus heated to 50° C was inferior to live virus, alumina-gel virus preparations were ineffective.

Perhaps the most hopeful observations yet made in regard to active immunization against poliomyelitis are those of Milzer, Oppenheimer, and Levinson They showed (1944, 1945) that suspensions of mouse brain and cord infected with the Lansing strain were inactivated in under one second by ultraviolet light Mice were vaccinated with 3 injections of 0.5 c c of irradiated suspension at weekly intervals, they showed a marked resistance to a subsequent intracerebral inoculation of living virus Loring *et al* (1947) have rendered cotton rats resistant to cerebral challenge by formalin-inactivated purified virus

SERUM ANTIBODIES IN EXPERIMENTAL POLIOMYELITIS

In Monkeys

Antibodies develop irregularly in monkeys on recovery from infection, and are not detected until the lapse of 2–3 months (Sabin and Olitsky, 1936 *a*, Harford and

some effect was noted (1943). When serum was given after infection, the maximum protection was noted at 6 hours, but some was shown at 96 hours (1943).

MECHANISM OF RESISTANCE TO INFECTION IN EXPERIMENTAL POLIOMYELITIS

It is probable that one mechanism underlying the resistance of immune animals is a definite hindrance to the spread of virus by nerve paths Thus Rasmussen and Clark (1942) found that on inoculation of the sciatic nerve, virus failed to spread to higher levels in immune animals

It appears, however, that when immune monkeys are challenged with virus some invasion frequently occurs, but does not progress far, and clinical evidence of infection is slight or absent. For example, when immune monkeys are infected by nasal instillation, changes occur in the CSF, but virus does not reach the central nervous system (Flexner, 1935 *a*, 1937; Sabin and Olitsky, 1938).

On intracerebral challenge, immune monkeys may develop a febrile reaction within 24–48 hours (Jungeblut, 1931), such animals may show typical lesions of varying extent and intensity, and yet fail to develop clinical signs of infection (Faber, Silverberg, and Dong, 1943 *a*). The same workers (1943 *b*) immunized cynomolgus monkeys by repeated application of virus to the mucosa of the upper respiratory tract and alimentary tract, changes developed in the corresponding peripheral ganglia but not in the CNS. When challenged intracerebrally, changes were found in the cerebrum and brain stem but not in the cord They concluded that these immune animals, as a result of surface exposure, were able to limit the spread of infection, but were not able to prevent the implantation and growth of virus. The immune state was probably associated with the continued presence of virus in the peripheral ganglia.

Further evidence for the theory that a considerable degree of invasion does occur in immune monkeys was afforded by the experiments of Howe and Bodian (see above) They showed that animals recovered from infection contracted by one route could be infected by another route, the second invasion might cause histological changes, but no extension of paralysis Howe and Bodian further emphasized that animals immune to homologous virus may be susceptible to an heterologous strain

The observations just described make it evident that the resistant state in immune animals is at any rate partly dependent on some alteration in susceptible nerve cells

possible adjuvant

but less success-

can influence the

course of infection There are, nevertheless, other observations suggesting that it plays no essential part in the resistant state, its presence may be regarded as an accompaniment, rather than the cause, of the resistant state

Thus, antibodies do not develop in monkeys for several weeks after recovery

is, animals are resistant to challenge (Junge-

n be stimulated by repeated peripheral injection of virus, yet only after numerous injections do the animals become resistant to cerebral challenge (Howitt, 1937 *b*). Resistance to a challenge dose is acquired considerably after the development of antibodies Kessel and Stimpert (1941) found a correlation between the presence of neutralizing antibody and the resistant state in only 50 per cent of monkeys. Lennette and Hudson (1939) came to a similar conclusion

Although serum antibody may well play only a small part in the mechanism of resistance to infection, recent work of Morgan (1947 *b*) on the presence of anti-

ANTIBODIES IN TISSUES IN EXPERIMENTAL POLIOMYELITIS

The relative distribution of antibodies in serum and nervous tissue has been studied by Morgan (1947 *b*). In vaccinated monkeys, no antibodies could be found in the spinal fluid or gray cord at a time when they were present in the serum. In marked distinction was the distribution of antibodies in monkeys paralyzed after cerebral injection, the highest titers of antibodies were found in the anterior horn of the spinal cord, serum and spinal fluid contained little or no antibody. Antibody in the anterior horn appeared between 11 and 16 days after the onset of paralysis, and persisted for at least 4–5 months, by this time antibody was present elsewhere, but in lower titer. The highest titers were found in the anterior horns, then the medulla; lower titers were found in the insusceptible amygdala and visual cortex, and little or no antibody was found in the white matter of the cord or brain.

PASSIVE IMMUNITY IN EXPERIMENTAL POLIOMYELITIS

In Monkeys and Chimpanzees

Human or monkey serum. Numerous authors have recorded the results of injecting human or monkey immune serum in experimental infection, some found that neither prophylactic nor curative injections of such sera have any beneficial effect (e g, Landsteiner and Levaditi, 1910, Abrahamson, 1916–19 *a*, Brodie, 1935 *c*, Gordon, Hudson, and Harrison, 1939, Harrison and Woolpert, 1939). Flexner and Lewis (1910 *e, f*), however, found that intrathecal injections of serum prevented the development of paralysis due to a dose of virus given 18 to 24 hours previously, similar effects were obtained by Flexner and Amoss (1917 *a*). Aycock and Amoss (1923) reported beneficial curative effects when serum was injected intrathecally and hypertonic saline given intravenously, preferably with more antiserum. Schultz and Gebhardt (1935) reported that, at best, a high titered serum might protect against a subsequent small dose of virus.

Monkeys passively immunized by the intravenous route showed no signs of infection following the intravenous administration of virus, starch being given intracerebrally at the same time (Lennette and Hudson, 1939). Lennette and Campbell (1939) found that horse serum regularly penetrated the blood-brain barrier of infected animals in small amount.

Chimpanzees protected by hyperimmune monkey serum became infected when stool was administered orally (Howe and Bodian, 1945 *b*).

Any change exerted by antiserum on the experimental disease is at best slight. No outstanding successes have been obtained which would encourage the use of serum therapy in man.

Horse or sheep serum. Various workers have reported that antipoliomyelitis horse serum has modified the experimentally produced disease, thus, injected intrathecally in the incubation period no paralysis developed (Weyer, Park, and Banzhaf, 1931). Others, however, have found the serum of little use (Rhoads, 1931 *b*, Howitt, 1934).

Pettit's serum has been said to show some prophylactic powers in monkeys against subsequent injections of virus (Howitt *et al*, 1931, Howitt, 1932). It is probably, however, of doubtful value clinically (Marinesco, Manicatide, and Draganesco, 1929, Howitt *et al*, 1931, Violle and Montus, 1935).

In Rodents

Kramer worked with the Lansing strain in mice and found that a significant proportion of 3-week-old animals was protected against an intracerebral injection, by the intraperitoneal administration of 0.03–5 c c of human convalescent serum (1941). This protective action of serum lasted for 72 hours, and even after 2 weeks

CHAPTER LXXXVIII

IMMUNITY IN HUMAN POLIOMYELITIS

ALLERGY

ALTHOUGH Jungeblut (1930–1 *c*) showed that convalescents reacted to the intradermal injection of heated virus filtrates with a definite erythema, others have been unable to detect any difference between convalescents and controls (Harmon, Harrison, and Kernwein, 1932–3, Sabin, Park, and Jungeblut, 1933).

ACTIVE IMMUNITY FOLLOWING INFECTION

A clear distinction should be drawn between what is implied by the terms "relapse" and "second attack" in poliomyelitis Relapses are most uncommon and occur within 3 months of the first attack It is possible that the virus is carried in the respiratory tract during the interval, as relapses have occurred after rhinopharyngitis and pertussis (Loeschke, 1934).

Second attacks occur from 2 to 16 years after the first, but are generally thought to be rare (e g, Francis and Moncrieff, 1919, Still, 1930, Moore, 1934, Quigley, 1934, Cohen, 1935, Fischer and Stillerman, 1938; Toomey, 1938 *c*; Nelson and Green, 1943; Wyllie, 1945, Lipscomb, 1947).

However, from their observations in Buffalo, Bridge, Clarke, and Abbe (1946) cast doubt on the existence of significant acquired immunity The whole question is in need of further study. Fundamental is the question whether or not there are several antigenic types of virus

It is certain that in countries where the disease is endemic, the majority of persons becomes immune to infection at a fairly early age. Some of these persons may have suffered from mild symptoms, but the majority has acquired resistance through being healthy carriers The intestine is probably a more important site of carriage than the nasopharynx. It is possible that enough virus is absorbed to stimulate the production of antibodies and cell resistance. It is possible, however, that the "immunizing" agent may actually act locally in the epithelial cells of the gastrointestinal tract by blockading them against later invasion (see Evans and Green, 1947)

ACTIVE IMMUNITY FOLLOWING VACCINATION

Two vaccines have been very widely used in America in an effort actively to immunize children against poliomyelitis, namely, Brodie's formolized and Kolmer's ricinoleated vaccines. A considerable amount of experimental work was carried out with these vaccines before they were used in human experiments (see p 1009)

Brodie's vaccine Brodie (1934–5 *a*) injected 12 children both intracutaneously and subcutaneously The inoculum consisted of 5 c c of a 10 per cent. virus suspension inactivated with 0 1 per cent formalin for 16 to 48 hours, 1 to 2 5 c.c. was injected intradermally and the remainder subcutaneously, no general or local disturbance was noted. After vaccination the children's sera showed an appreciable increase in the antibody titer. Brodie (1935 *a*) found that 0 05 per cent formalin was as effective

Later, Brodie and Park (1935 *b*) reported the results of over 2,300 vaccinations with formalized virus, in which only 17 children showed local reactions, and no serious accident occurred A rise in the neutralizing antibody titer of the serum was noted beginning after 1 week, reaching its height in 3 to 4 weeks

Kramer (1936 *a*) vaccinated a small group of children with Brodie's vaccine. Some 2 months later 50 per cent had developed neutralizing antibodies, but of a *parallel* uninoculated control series 41 per cent. had also developed antibodies Aycock and Hudson

body in the anterior horn a few days after the onset of paralysis, throws new light on the subject (see p. 1012). She suggests that this local antibody may prevent further infection, if the challenge is given by the same route. This may correlate with the observations of Howe and Bodian (above) that previously uninvaded paths in resistant monkeys may become infected with virus when the animals are inoculated by routes different from the original. It may be that this is possible because of a relative absence of antibody.

OCCURRENCE OF SERUM ANTIBODIES

Normal Persons

The term "normal" in connection with poliomyelitis signifies persons who have never suffered from any recognizable manifestation of infection, nor have been in close contact with a case, so far as they are aware. It is well nigh impossible, however, to be certain that so-called normal persons have never suffered from abortive (nonparalytic) poliomyelitis or been carriers Numerous [illegible] orded the presence of virus neutralizing [illegible] rmal persons (e [illegible] 1929–30, Aycock [illegible] Gebhardt, 1930–1 [illegible], Netter, Levaditi, and Hornus, [illegible] 1935 a, Paul and Trask, 1935, Brodie, Fischer, and Stillerm[illegible], 1937, Howitt, 1937 b).

The sera of many normal adults neutralize several strains of virus (Aycock, 1942 c)

Age distribution of immune bodies Young infants receive passive immunity from their mothers, and tests carried out on maternal sera and cord bloods show a complete correspondence between the presence of antibodies in mother and child, approximately 80 per cent. of mothers' and babies' sera neutralize the virus (Aycock and Kramer, 1930 *a*, *c*).

The results of various examinations of neutralizing antibody in children's sera at different ages have been compiled of children aged 0 to 4 years, 37 per cent. show the presence of immune bodies, from 10 to 14 years the sera give 58 per cent. of positive readings, while over 15 years 73 per cent. of sera neutralize the virus (Harmon and Harkins, 1936). More recently, antibody tests have been carried out in mice, and have confirmed the earlier observations that the bulk of adult sera neutralizes the virus, and that the percentage of sera neutralizing increases with age (Haas and Armstrong, 1940, Armstrong and Davis, 1945; Turner, Young, and Maxwell, 1945, Brown and Francis, 1947 *b*) There is thus a steady increase in the percentage of sera neutralizing the virus with advancing age.

Geographical distribution of immune bodies The various authors already mentioned examined sera from parts of the world (especially the United States) where poliomyelitis is common. Sera have also been examined from parts of the world where epidemic poliomyelitis is less common. Thus, the sera of 18 19 Liberian (African) males over 15 years of age neutralized the virus (Hudson and Lennette, 1933) The sera of 3 adult Eskimos and 11 Chinese all showed positive neutralizing power (Jungeblut, 1933), as did another series of 8 10 Chinese sera from Peiping (Lennette and Hudson, 1932–3) The sera of 8 adults from Porto Rico also neutralized the virus (Soule and McKinley, 1931–2). The sera of 9 10 Filipinos over 12 years of age neutralized the virus (Doull, Hudson, and Hahn, 1935) Hudson and Lennette (1938) reported another such series of tests. They found that the majority of sera of persons from Thursday Island (Australia), Formosa, and Brazil neutralized the virus As poliomyelitis only occurs sporadically in these areas, they suggested that this result was evidence for the wide distribution of poliomyelitis virus

More recently, similar results have been obtained using strains such as the Lansing as antigen, in neutralization tests in mice. Thus antibodies have been found very commonly in the sera of "normal" West Africans (Findlay, Anderson, and Haggie, 1946, Olitsky and Findlay, 1946)

It is evident therefore that the serum of "normal" adults in most parts of the world neutralizes the virus The antibody in normal sera cannot be distinguished in its properties from that in convalescent sera (see, e g, Harrison and Hudson, 1940)

(1936) recorded a similar rise in titer in vaccinated persons' sera, but no more than occurred in a control uninoculated group.

A field trial of this vaccine was made by Gilliam and Onstott (1936) during a poliomyelitis outbreak in North Carolina and Virginia, 458 persons were inoculated with vaccine and 676 controls were used However, not a single case of poliomyelitis developed in the controls, so no opportunity was presented to test the efficacy of the vaccination

With regard to the safety of Brodie's vaccine, Brodie and Park (1935 *b*) found that 5 c c of virus inactivated by 0 1 per cent formaldehyde for 8 to 12 hours was noninfective for monkeys It should be remembered, however, that Trask and Paul (1936) found that 0 1 per cent. formalin did not destroy the virus in 24 hours in the refrigerator

Although no cases of poliomyelitis appear definitely to have been attributed to the use of Brodie's vaccine, some hesitancy should be shown in regarding it as avirulent

Kolmer's vaccine With regard to any type of vaccination, it may justifiably be asked whether it is worth while, as so very few children actually contract the infection, compared to the number at risk Kolmer (1935 *b*) has given his justifications for embarking on a scheme of vaccination (1) The active immunity induced by the natural disease is so efficient that it was hoped vaccination might accomplish the same effect (2) Although the attack rate is low the disease seems to be on the increase (3) The sera of approximately 80 per cent of children under 4 years of age do not contain any appreciable quantity of antibody (4) Although only 25 per cent. of children over 15 fail to show virus neutralizing bodies, this process of active immunization should not be regarded as adequate protection (5) Monkeys could be immunized with ricinoleated virus (6) Ricinoleated vaccine stimulated the production of antibodies in man, which appeared even after 4 days, and no person receiving 3 doses contracted poliomyelitis, although some persons receiving 1 to 2 doses did

Kolmer's vaccine differs from Brodie's in that it is definitely known to contain active virus Kolmer and Rule (1937), working with monkeys, found they could not produce active immunity unless there was enough virus in 0 3 c c of vaccine to produce paralysis on intracerebral injection Kolmer has attached some importance to the fact of the virus being monkey passage strain and, therefore, in his opinion, of lowered virulence for man As events have shown, the virus retained sufficient virulence to infect some of those vaccinated, even by the subcutaneous route

A preliminary batch of 25 children was injected subcutaneously with a 4 per cent cord suspension in 1 per cent. sodium ricinoleate (Kolmer and Rule, 1934 *a*, *b*, Kolmer, Klugh, and Rule, 1935 *a*, *b*; Kolmer, 1935 *a*). The vaccine was prepared by emulsifying 8 gm. of monkey cord in 100 c.c. of sterile saline, after fine mesh filtration, equal parts of 2 per cent. sodium ricinoleate in saline were added (giving a final strength of 4 per cent cord in 1 per cent ricinoleate) The mixture was incubated at 37° C for 24 hours, and thereafter kept in the refrigerator at 4° to 6° C for 14 days, being shaken occasionally. In certain cases phenylmercuric nitrate or mercurophen was added (Kolmer, 1936 *b*) Three injections were given at intervals of 7 to 10 days No serious disturbance was noted although there might be some local tenderness at the site of inoculation

Antibodies appeared, even as early as after 4 days, in those who failed to show them before the treatment, in other cases the preexisting titer was increased Repeating this work, Aycock and Hudson (1936) showed that antibodies appeared after vaccination, but not in any higher percentage than occurred in a control unvaccinated group

Encouraged by this preliminary success, Kolmer and Rule (see 1937) sponsored the inoculation of some 10,725 individuals in 36 different States between April and September 1935 Injections were given subcutaneously and no case receiving 3 full doses contracted poliomyelitis Unfortunately, however, 10 cases of poliomyelitis followed in those receiving fewer doses Further issue was refused (Kolmer, 1936 *a*)

Leake (1935, see also 1936 *b*) recorded some 12 cases of paralysis following injection with 2 unnamed vaccines, presumably at least 10 of these cases arose following Kolmer's vaccine The level of the spinal cord first affected usually corresponded to the injected extremity, suggesting a direct neural spread of the virus At the time of writing, Leake recorded that 6 of these cases had died

It is unlikely that active immunization with attenuated virus will be applied to children again (see Kolmer, 1938), although suggestions have been made that a possible line of approach to the problem is oral immunization with a so-called nonparalyzing strain

tion by a single strain, and found that the majority of convalescent sera gave a specific response with this strain

Convalescents from Abortive Attacks

A number of workers has shown that abortive attacks of poliomyelitis give rise to the production of antibodies in convalescence (see, e g, Netter and Levaditi, 1910 *b*, Anderson and Frost, 1911, Howitt, 1932, Eagles, Jensen, and Henningsen, 1937, Gildemeister, 1939).

Virus Neutralizing Properties of Body Fluids

Policidal properties have been found in the normal nasopharyngeal secretion (*Amoss and Taylor, 1917*), *the nasal secretion* (Howitt, 1937 *b*), the tears (Jungeblut, 1934–5) This poliocidal substance is probably of a lysozym nature. The cerebrospinal fluid of convalescents neutralizes the virus, even after 18 months (Meyer and Le Guyon, 1932, Meyer, 1934), probably due to a true virucidal antibody.

SIGNIFICANCE OF THE WIDESPREAD DISTRIBUTION OF ANTIBODY

About 75 per cent of supposedly normal persons' sera neutralize poliomyelitis virus This can most readily be explained on the basis of widespread subclinical infection However, *two alternative theories have been advanced*

The Theory of Infection

This theory is orthodox, and affirms that the presence of virus neutralizing antibody is due to previous contact with poliomyelitis virus It is now known that poliomyelitis virus enjoys a widespread distribution It is thought to occur in practically every country, and to infect a very much larger number of persons than actually suffers from clinical evidence of infection The majority of persons is thought to become infected in childhood, probably through close contact with a *case, paralytic or abortive Such persons probably harbor the virus in the naso*pharynx for a few days, and in the intestines for a few weeks This relatively short period of carriage is sufficient to give rise to active immunity characterized by the development of resistance to infection and of serum antibodies This view, confirmed by recent work on the epidemiology of the disease (Ch LXXXIII was supported on statistical grounds by Stocks (1932) He believed that in endemic areas, for every 1 person showing paralysis, over 100 acquired resistance by latent epidemization.

The Theory of Physiological Maturation

Jungeblut has been largely responsible for criticizing the infection theory, and propounding an alternative (Jungeblut, 1934, 1935–6, 1936 *b*, 1937 *a*, *b*, Jungeblut and Engle, 1931–2, 1932 *a*, *b*, 1933, Jungeblut, Meyer, and Engle, 1934, Jungeblut

1 Even on its original appearance in a country, the disease has always selectively attacked the prepubertal child (contrast, e g, measles epidemics in virgin soil) If immunity is dependent upon subclinical infection, then all ages should be susceptible, as adults have had no opportunity for previous contact with the virus.

CONTACTS

It has been stated that the sera of those with a history of frequent exposure to infection have a higher immune body content than those without such obvious contact (Brodie, 1932 *c*; Brodie and Park, 1935 *a*). Kramer (1932) examined a group of persons who were in intimate and prolonged contact with a case of poliomyelitis At the beginning of the experiment, these persons showed no immune bodies, whereas after 6 months they had developed them, although they never showed any signs of abortive attacks of the disease. A control group of persons who came into less intimate contact did not develop immune bodies. However in the 1937–8 Victoria epidemic Burnet and Jackson (1939) did not find that contacts developed antibodies.

CONVALESCENTS FROM PARALYTIC ATTACKS

The development of immune bodies during convalescence from frank paralytic attack has been noted by many (e g, Netter and Levaditi, 1910 *a*, Trask and Paul, 1933, Brodie, 1934 *c*, Harmon *et al.*, 1936, Brodie, Fischer, and Stillerman, 1937). Estimates of the time of first appearance of these bodies vary from the 3rd day after onset of paralysis (Kling and Levaditi, 1913 *b*) to the 9th to 14th day (Harmon *et al.*, 1936).

In general, samples of convalescent sera are more strongly virucidal than normal sera (see Aycock, Luther, and Kramer, 1929; Southby and McKie, 1933; Brodie, 1934 *c*, Kessel, Stimpert, and Fisk, 1939). However, certain normal samples may show a titer which is quite as high as, if not higher than, this It has been stated that 0.04 c.c. of a strongly virucidal convalescent serum should neutralize 80 m.c p. doses of virus (Brodie, 1934 *c*)

Until fairly recently, it was assumed that the majority of convalescent sera showed the presence of virus neutralizing properties, but Harmon and Harkins (1936) compiled the results of a number of such examinations, and disclosed that of 183 convalescent sera tested, 39 8 per cent gave a negative result. Later observations have served to confirm this view (Gildemeister, 1939, Burnet *et al*, 1939, Burnet and Jackson, 1939, Burnet, 1940, Turner and Young, 1943) Thus, Burnet and his colleagues investigating a number of cases in the 1937–8 Victoria epidemic found no rise in titer when acute phase samples were compared with convalescent samples obtained 2–3 months later, they used both the MV and a local strain as antigen. Turner and Young (1943) used the Lansing strain as antigen and carried out the titrations in mice They found that in 52/64 cases, the acute and convalescent phase samples gave the same results. They found only one patient who showed a complete antibody response in convalescence Brown and Francis (1947 *b*) using the Lansing strain, only detected a rise in antibody titer in 3/74 convalescents

Burnet, Turner, and Young, and Brown and Francis drew attention to the fact that many of their patients had neutralizing antibody in their blood at the onset of the disease. Hammon and Izumi (1942 *a*) found that about 50 per cent of sera in the acute phase neutralized the Lansing strain. The question of the virus used as antigen in these tests is of primary importance, in view of the antigenic differences known to exist between virus strains Burnet used a local strain, so his results definitely point to a failure of development of antibody However, Turner and Young,

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as yet. Aycock (1942 *c*) has pointed out that apparent discrepancies in the neutralization test may be explicable by the fact that different strains may be present in a single epidemic. He studied an outbreak where the epidemiology pointed to infec-

they caused immature monkeys to menstruate by injecting them with anterior pituitary extract, but no immune bodies developed

Physique. Jungeblut draws attention to the descriptions given by Draper (1932, 1935) of the typical poliomyelitis child (see p. 919), all indicating some widespread endocrine disturbance. However, these stigmata are not accepted as specific by Levine, Neal, and Park (1933).

Ablation of endocrine glands The sera of Nicaraguan cebus monkeys were found to ne [illegible] -2), after adrenalectomy, however, they [illegible] ortical hormone the neutralizing power [illegible] and Steinbach (1934–5) also found that [illegible] of natural poliomyelitis antibody. The antibody present in animals recovered from infection was not so affected by adrenalectomy. Removal of thyroid or testicle, or the injection of thyroxin had no effect on the refractory state of monkeys recovered from infection (Levaditi and Haber, 1935).

Aycock (1936, 1937) castrated immature female rhesus monkeys and treated them with estrin Although the numbers were small, it appeared as if the estrin-treated animals were more resistant to nasal inoculations of virus than castrates who had received no estrin, or normal controls (see also Curley and Aycock, 1946).

The effect of vitamins It was found that if monkeys injected with virus intracerebrally were treated with repeated doses of vitamin C (5 mg) 50 per cent. survived If the dose of vitamin was considerably increased, the results were not so good, the majority of animals succumbing (Jungeblut, 1937 *a*) Later, Jungeblut (1937 *b*) reported that 32 per cent. of monkeys injected intracerebrally survived without paralysis when treated with natural vitamin C. Treated with synthetic vitamin only 10 8 per cent survived (see also Jungeblut, 1939). Sabin (1939) reported, however, that vitamin C had no effect on the course of infection after intranasal instillation of virus Continuing these researches, Jungeblut and Feiner (1937) investigated the vitamin C content of the tissues of monkeys infected with poliomyelitis They found the content slightly below the average in the adrenals and nervous tissue of paralyzed monkeys (treated or not with vitamin), the content of the organs of treated, nonparalyzed monkeys was markedly higher. Kasahara and Gammo (1938), however, found no change in the vitamin C content of the cerebrospinal fluid in experimental poliomyelitis

Autarceologic susceptibility This term has been used by Aycock (1929, 1930, 1941) While not de [illegible] the virus, Aycock h[illegible] logic susceptibility " [illegible] the normal physiological functions of the body. He attaches great importance to seasonal variations giving rise to physiological imbalance and resulting susceptibility to infection. Aycock thus supports Jungeblut in his claims that physiological mechanisms are responsible for resistance to poliomyelitis Aycock's (1936, 1937) work with castrated monkeys has already been mentioned He holds that susceptibility to poliomyelitis may depend on some pituitary gonad imbalance.

Conclusions Jungeblut (1937 *a*) has formulated the following expression of his views Poliomyelitis is essentially a developmental error of youth in which protection is due to physiological factors. Man is normally insusceptible and can carry the virus with impunity, the disease only occurring in a few, and this due to some transient physiological abnormality

The Theory of Nonspecific Infection

It has been suggested that the presence of poliomyelitis antibody is due to infection with antigens chemically sufficiently close to the virus to provoke an antibody response similar to that provoked by the virus itself. There is little or no evidence in favor of this theory with one exception some relative data have been

2. Neither paralytic nor abortive cases are common enough to explain the widespread distribution of immune bodies

3. Poliomyelitis can occur in persons who have been exposed to infection for such a length of time that resistance might reasonably be expected to have developed

4. In the United States the same towns are frequently revisited by epidemics in later years, but the inhabitants do not appear to have become immunized.

5 Jungeblut states that there is no evidence that a subinfective dose of virus can provoke immunity, citing his own experiments in which no immunity followed repeated spraying of a monkey's nasopharynx (Jungeblut and Hazen, 1930–1 *b*)

6 It has been mentioned already that the sera of Filipinos, Porto Ricans, Liberians, Eskimos, and Chinese neutralize the virus As poliomyelitis is rare in the lands inhabited by these people, it is argued that the immune body may have been produced in the absence of the specific virus

7 It may be mentioned here that neutralizing antibody has been found in the sera of normal pregnant mares (Jungeblut, Meyer, and Engle, 1934, Jungeblut, 1935–6). Normal cebus and rhesus monkeys' sera also neutralized the virus (Jungeblut and Engle, 1931–2)

Jungeblut and his colleagues believe that immunity to poliomyelitis is dependent on investigated a number of possibilities

One of the earliest reports on the blood gro hat of *Grooten and Kossovitch (1930)*, who noted that a higher percentage of poliomyelitis patients belonged to group A than did controls, slightly fewer poliomyelitis patients belonged to group B and O than would be expected, and none at all to AB

Jungeblut and Smith (1932) made a more extensive investigation They noted that below 5 years of age the incidence of blood groups in paralytic cases was perfectly normal. With advancing age, however, there was a diminution in those belonging to B and a slight increase in O After 5 years of age most deaths occurred in groups other than B, particularly A (5 to 10 years) or O (over 10 years)

With regard to blood donors, they noted that the sera of 33 per cent of A, 50 per cent of O and AB, but 80 per cent. of B groups neutralized the virus These figures were confirmed by Jungeblut (1933), who examined the sera of New Yorkers who had lived there through the 1931 epidemic, he made the additional observation that when these sera were titrated, O neutralized virus diluted 1 20, A, 1 5, but B neutralized virus diluted even 1 60

The subject attracted some interest and a number of other papers has appeared 1933, Cowie, Parsons, and Lowenberg, 1934–5, vhether any significance can be attached to the question of the varying incidence of poliomyelitis in different blood groups following the work of Madsen, Engle, Jensen, and Freuchen (1936). These workers investigated the blood groups of approximately 1,200 patients and 20,000 normals *during the 1934 Danish epidemic They concluded that there was no significant* difference in the incidence of cases occurring in each group, when contrasted with controls More recently, Erb *et al* (1938) studied the blood groups *in 703 cases* during the 1937 autumn epidemic in Toronto, they found that susceptibility to infection bore no relation to blood grouping. With regard to the greater virus neutralizing power claimed for group B sera, further observations are required, if possible on a large scale

Menstruation The immune body content of the sera of two women varied, on one occasion giving a positive neutralization test, and on another a negative (Jungeblut and Engle, 1933), indicating that physiological mechanisms may affect titer

Similarly, it was found that the sera of 2 monkeys examined before the onset of the menstrual cycle failed to neutralize, reexamined 10 days after the onset of bleeding, a positive result was obtained (Jungeblut and Engle, 1931–2) A more extensive study was carried out by Hudson, Lennette, and King (1934) who found that sera taken at various stages of the menstrual cycle failed to neutralize Further,

they caused immature monkeys to menstruate by injecting them with anterior pituitary extract, but no immune bodies developed.

Physique. Jungeblut draws attention to the descriptions given by Draper (1932, 1935) of the typical poliomyelitis child (see p. 919), all indicating some widespread endocrine disturbance. However, these stigmata are not accepted as specific by Levine, Neal, and Park (1933)

Ablation of endocrine glands. The sera of Nicaraguan cebus monkeys were found t ... after adrenalectomy, however, th ... hormone the neutralizing po ... Steinbach (1934–5) also found t ... tural poliomyelitis antibody. ... nfection was not so affected by adrenalectomy. Removal of thyroid or testicle, or the injection of thyroxin had no effect on the refractory state of monkeys recovered from infection (Levaditi and Haber, 1935).

Aycock (1936, 1937) castrated immature female rhesus monkeys and treated them with estrin. Although the numbers were small, it appeared as if the estrin-treated animals were more resistant to nasal inoculations of virus than castrates who had received no estrin, or normal controls (see also Curley and Aycock, 1946).

The effect of vitamins It was found that if monkeys injected with virus intracerebrally were treated with repeated doses of vitamin C (5 mg.) 50 per cent. survived. If the dose of vitamin was considerably increased, the results were not so good, the majority of animals succumbing (Jungeblut, 1937 *a*). Later, Jungeblut (1937 *b*) reported that 32 per cent. of monkeys injected intracerebrally survived without paralysis when treated with natural vitamin C. Treated with synthetic vitamin only 10 8 per cent. survived (see also Jungeblut, 1939). Sabin (1939) reported, however, that vitamin C had no effect on the course of infection after intranasal instillation of virus. Continuing these researches, Jungeblut and Feiner (1937) investigated the vitamin C content of the tissues of monkeys infected with poliomyelitis They found the content slightly below the average in the adrenals and nervous tissue of paralyzed monkeys (treated or not with vitamin); the content of the organs of treated, nonparalyzed monkeys was markedly higher Kasahara and Gammo (1938), however, found no change in the vitamin C content of the cerebrospinal fluid in experimental poliomyelitis

Autarceologic susceptibility This term has been used by Aycock (1929, 1930, 1941) While not denying the importance ... the virus, Aycock has stressed the rôle of ... logic susceptibility " Autarcesis is the powe ... the normal physiological functions of the body. He attaches great importance to seasonal variations giving rise to physiological imbalance and resulting susceptibility to infection Aycock thus supports Jungeblut in his claims that physiological mechanisms are responsible for resistance to poliomyelitis. Aycock's (1936, 1937) work with castrated monkeys has already been mentioned He holds that susceptibility to poliomyelitis may depend on some pituitary gonad imbalance.

Conclusions Jungeblut (1937 *a*) has formulated the following expression of his views Poliomyelitis is essentially a developmental error of youth in which protection is due to physiological factors Man is normally insusceptible and can carry the virus with impunity, the disease only occurring in a few, and this due to some transient physiological abnormality.

The Theory of Nonspecific Infection

It has been suggested that the presence of poliomyelitis antibody is due to infection with antigens chemically sufficiently close to the virus to provoke an antibody response similar to that provoked by the virus itself. There is little or no evidence in favor of this theory with one exception. some relative data have been

obtained in the case of diphtheria. Thus monkeys immunized against diphtheria may resist infection with poliomyelitis virus (Armstrong and Harrison, 1933) Their sera may neutralize the virus *in vitro* (Jungeblut, 1934, 1936*a*). Zingher (1917*a*) found that 80 per cent. of children convalescent from poliomyelitis gave a positive Schick test, as against 30 per cent. of normal children; Dwyer (1938) found that Australian children immunized against diphtheria were less likely to contract poliomyelitis than unimmunized children. Brodie and Park (*1935 a*), however, using the Schick test, could find no relationship between immunity to diphtheria and poliomyelitis.

This theory has received support from Burnet (Burnet and Jackson, 1939, Burnet *et al.*, 1939). He concluded that poliomyelitis antibody is not the result of *exposure to, or infection with, the virus of poliomyelitis.* It is produced in response to another or [illegible] opounded by Burnet is that the an [illegible] ociated with increasing age, and [illegible] correlation between the presence [illegible] n in young children from industrial districts, and suggested they were both acquired at the same age and in the same environmental circumstances.

Conclusions

We do not feel that a sufficient case has been made out against the orthodox view that antibody results from exposure to the virus, as is accepted without question in other virus diseases. The work of the last few years has made it increasingly evident that poliomyelitis virus is, in fact, a widespread infecting agent in man

Thus, the principal difficulty of earlier workers in accepting the infection theory *seems largely to have disappeared. Further observations are required on the antibody* response of close contacts who develop infection and excrete virus

IMMUNE SERUM THERAPY IN HUMAN POLIOMYELITIS

One of the most practical points to discuss in this chapter is the question of the administration of immune serum in the prevention and treatment of poliomyelitis, and as an introduction it is as well to consider [illegible] the use of serum is based on the assumption that [illegible] fect on the virus *in vivo* However, immune s[illegible] effect on the experimental disease (*see above*). *Further, it has already been mentioned* that it is now doubtful whether the antibody has any essential significance in resistance to infection.

In the second place, when serum therapy was introduced over 30 years ago by Netter, Gendron, and Touraine (1911), it was generally held that poliomyelitis showed 2 distinct stages of infection. At first the virus was thought to circulate in the blood, and only later did it localize in the meninges According to this view, the use of serum was quite rational, as there was some likelihood of virus in the blood being neutralized Even later, the injection of serum into the spinal theca might be hoped to neutralize virus in the meninges and prevent the development of paralysis.

As mentioned on many occasions in these chapters, current views on the pathogenesis of poliomyelitis differ widely from those in vogue 20 to 30 years ago Thus, it is now believed that virus is present in the central nervous system from the beginning, and that no stage of primary blood involvement occurs Now, if the virus is present in the axons it is unlikely that serum will be able to influence *it at all* readily At the outset, therefore, it appears that there is little justification for expecting serum therapy to be of much avail, for even if the antibody has any action *in vivo*, it is difficult for it to reach the virus The examination of carefully controlled series of administrations of serum supports the modern view, as it is gen-

erally agreed that such observations have failed to detect any benefit in serum-treated cases.

Animal Serum

Pettit (1935) has advocated the use of animal sera, and claims that, if given in the preparalytic stage, paralysis is prevented If given later, paralysis may be cured, or spread prevented (see also Casassa, 1938). The bulk of opinion is, however, against the use of heterologous sera, and these results must be accepted with some reserve.

The Choice of Human Serum

Three different types of donor are commonly used to supply poliomyelitis immune serum: convalescents from paralytic attacks, convalescents from abortive attacks, and normal individuals.

Donors convalescent from paralytic attacks. This source of supply is probably

Brodie, 1934 c)

Donors convalescent from abortive attacks The sera of these convalescents may show as high a percentage of antibodies as those convalescent from paralytic attacks, and so may be used in therapy (Anderson and Frost, 1911, Howitt, 1932, Eagles, Jensen, and Henningsen, 1937, Heck, 1942)

Normal sera Normal sera may show virus neutralizing bodies in high concentration, and it has been recommended that ordinary blood transfusion donors be used, and that their serum be tested for immune body as well as for blood grouping (Faber, 1931)

Pooled sera. When large quantities of immune serum have to be available, it is obviously simplest to pool all the supplies As the immune body content of different samples is somewhat variable, the titer of pooled sera is usually low Strictly speaking, all sera to be used in therapy should be subjected to a virucidal test before being pooled, and those of low titer discarded (see, e g, Faber, 1931) To minimize the risk of serum jaundice, pools should not consist of more than 10 samples

International standard serum Madsen and Jensen (1936) have prepared a standard antiserum which is available for use throughout the world After the cessation of the 1934 Danish outbreak, some 300 liters of serum which remained were desiccated and the dry powder carefully mixed, 500 gm of dry powder were dissolved in 5 liters of distilled water This was then centrifuged at 10,000 r p m for 1 hour, filtered through a Seitz, tested for sterility and distributed in 5 c c amounts in nitrogen-filled ampules

The use of whole blood in poliomyelitis Intramuscular injections of 60 c.c. of parents' whole blood were recommended by Stokes *et al* (1935). Later it was reported that unpleasant reactions might occur if the cells were incompatible (Rapoport and Stokes, 1937)

Gamma globulin Bahlke and Perkins (1945) gave 20 c c intramuscularly to those under 1 year, and up to 100 c c in the case of those of 12 and over There was no benefit

Route of inoculation The serum is most commonly given intrathecally with or without an accompanying intravenous injection Intrathecal injection provokes a polynucleosis in the spinal fluid, and this may itself *prove beneficial* (Sophian, 1916, Zingher, 1917 *b*) The accompanying meningeal irritation, however, increases the neck stiffness and Kernig's sign (Aycock, Luther, and Kramer, 1929). It may also be administered subcutaneously or intramuscularly

Time of inoculation Although some of the earlier workers (e g, Netter, 1915) claimed that the serum benefited paralyzed cases, for some time now its use has

been more or less restricted to the preparalytic stage in the hope of aborting the onset of paralysis.

Dose. In the earlier days about 10 c.c. used to be given on several occasions More recently workers have used doses of 20 c.c or more intrathecally, combined with 50 c.c. intravenously, up to 50 c c. can be given intramuscularly.

A valuable adjunct is to inject hypertonic saline intravenously as well as the serum, thus tending to lower intracranial tension and lessen edema and pressure (Aycock and Amoss, 1923).

Preservation and storage. The serum may be heated to 55° C. for half an hour, or treated with 0 2 per cent. tricresol (Amoss and Chesney, 1917) or 0.2 per cent. phenol (Brodie, 1934 *c*), but a better method would appear to be that of Morgan

Morgan's method. This method has been used in Australia (Morgan, 1927, 1928, Burnet and Macnamara, 1929, Macnamara, 1929, Macnamara and Morgan, 1932)
Blood is withdrawn into oxalate and after c [illegible]
calcium chloride is added to cause clotting [illegible]
filter (EK), and after a second filtration, [illegible]
into ampules This product is free from ar [illegible]
children

Another convenient method of storage would be to desiccate, as used by Madsen and Jensen (1936). After one year's storage of serum preserved with 0 2 per cent. phenol, only 20 per cent of the neutralizing power was lost (Brodie, 1934 *c*) After 2 years' storage, 50 per cent had been lost, and finally after 4 years', only 25 per cent. of the original neutralizing power remained. Burnet and Macnamara (1929) reported that serum sealed in ampules without antiseptic could be stored for 3 years without significant loss of properties

Results: (a) relatively uncontrolled.

Numerous authors from all parts of the world have used immune serum, and it must be admitted that many have expressed satisfaction, they have reported the prevention of paralysis, falls of temperature, and clinical improvement dating from the inoculation Poliomyelitis is a capricious disease and unfortunately very few of the authors to be mentioned have carried out adequate controls (Netter, Gendron, and Touraine, 1911; Netter, 1915; Amoss and Chesney, 1917, Fischer, 1917, Shaw, Thelander, and Fleischner, 1925; Aycock, Luther, and Kramer, 1929, Marinesco, Manicatide, and Draganesco, 1929, Macnamara, 1929, Faber, 1931, Levinson, McDougall, and Thalhimer, 1932, Macnamara and Morgan, 1932, Petter, 1933, Pohlen, 1934, 1936 *a*, Jensen, 1934–5, Hegler, 1936, Jackson, 1937, Kauders, 1937 *b*, Cadham, 1942)

Harmon (1934) has published a lengthy article which should be read by all interested in this subject He analyzes the published reports of some 4,400 treated and 2,660 untreated cases He stresses the failure of statistical presentation to demonstrate the value of serum, and finds that the outcome in treated preparalytic cases does not differ from the average for untreated patients He allows, however, from clinical observations that the serum may have some value

(b) Controlled.

The great majority of published reports has not been free from the criticism that there were insufficient controls, and in fact in many series there were none at all Numerous authors have expressed dissatisfaction with the use of serum, but again these results were often not well controlled, and carried little weight The most important controlled observation on the value of serum, given in the preparalytic stage, was carried out in New York in the 1931 epidemic by the Health Department and the Academy of Medicine, the results were reported by Park (1932) and Fischer (1934) Thus, Fischer reported on 447 treated and 102 control patients

The outcome for the treated persons was considered to be no better, if as good, as for the controls This report, in association with recent experimental work, will doubtless considerably modify current practice in many parts of the world where serum is at the moment used freely. Of recent years, several other unfavorable reports have appeared. For instance, during the 1936–7 Memphis (Tenn.) outbreak, serum was administered to preparalytic and paralytic cases, and a control group was left untreated (Levy, 1938). *Although the total numbers were not large, it was* perfectly definite that serum exerted no beneficial effect. Serum was administered to test, and withheld from control, groups in the 1937 Ontario epidemic (Hyland *et al.*, 1938). The incidence of paralysis and death was essentially similar in the two groups; Silverthorne, Hawke, and Brown (1941) came to a similar conclusion. Mai (1938) carried out an adequately controlled series of administrations in Bavaria and found no evidence that serum had any value. Athenstaedt (1938), from a review of over 3,000 treated and 555 untreated cases, concluded that serum was of no value Bahlke and Perkins (1945) found gamma globulin to be of no value.

World use of serum Serum has been used on a large scale mainly in the United States, Scandinavia, Germany, and Australia, but is now much less popular

In the 1933–4 Danish epidemic some thousands of cases were treated On clinical [illegible] vas [illegible] acy [illegible] of [illegible] *6 a;* [illegible] ate [illegible] as [illegible]

In conclusion, therefore, the accumulated evidence is against the value of immune serum therapy, and in a review of the subject Faber (1941) concluded that there was little to support the belief that it could be curative.

MECHANISM OF RESISTANCE IN HUMAN POLIOMYELITIS

Until lately the view was fairly generally held, in poliomyelitis as in other infections, that immune body played an important rôle in resistance to infection Thus, convalescents as well as normal adults were presumed to be resistant largely because their sera contained antibody For example, it was suggested that effective resistance to infection was present if 0.5 c.c. of serum neutralized at least 10 minimum infecting doses of virus (Kolmer and Rule, 1935 *a*). *It is not now believed that poliomyelitis antibody plays any primary rôle in determining resistance to* infection Poliomyelitis can occur in persons with neutralizing antibody in the blood Antiserum is of no value in preventing the onset of paralysis. Further, experiments have rendered it evident that in the monkey resistance to infection is dependent on some alteration in the susceptibility of the nervous tissues, and not on the presence of antibody Schultz (1936) regards immune body as an adventitious by-product Some virus escapes into the blood and tissues and stimulates the production of antibody Antibody is an index of the attack of virus on nerve cells, and does not give protection The resistance enjoyed by normal adults or convalescents is alike attributable to previous contact with the virus, sufficient to render the nerve cells insusceptible In man as in the monkey, it is almost certain that some invasion by virus occurs in resistant persons, yet this does not progress far enough to cause serious lesions

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SECTION 10. VIRUS MENINGITIS

CHAPTER LXXXIX

ACUTE LYMPHOCYTIC CHORIOMENINGITIS

In 1934, Armstrong and Lillie, investigating St. Louis encephalitis by inoculating monkeys, isolated a virus that they called the "virus of lymphocytic choriomeningitis" (LCM). In addition to causing infection in mice and monkeys, numerous workers have isolated Armstrong's virus from cases of benign "aseptic" meningitis, and there is no doubt that it is the commonest individual cause of this condition (Armstrong and Wooley, 1935, Rivers and Scott, 1935, 1936, Scott and Rivers, 1936, Findlay, Alcock, and Stern, 1936, Barker and Ford, 1937, Lépine and Sautter, 1938, Armstrong and Sweet, 1939, Howard, 1939, MacCallum and Findlay, 1939, Findlay, Stuart-Harris, and MacCallum, 1940). However, the virus is not the sole cause of aseptic meningitis, and a similar clinical syndrome can occur in bacterial and other viral infections (Eckstein, Holtinger, and Schlerssing, 1932; Schneider, 1935; Rivers and Scott, 1936, Armstrong and Wooley, 1937, Baird and Rivers, 1938, Christie, 1943, Avery, 1945, Klopstock, 1945). The viral infections in which the syndrome of aseptic meningitis may occur include: mumps, measles, varicella, herpes zoster and febrilis, poliomyelitis, lymphogranuloma, and sandfly fever. A similar syndrome may occur in glandular fever (Tidy, 1946), hepatitis, and swineherd's disease (see below).

Many authors have published clinical descriptions of cases of aseptic meningitis, and some at least of these have been additional examples of infection with the virus of LCM (Wallgren, 1925, 1926, 1927, Viets and Watts, 1929, 1934, Dereux, 1931, Eckstein, 1931, Gibbens, 1931, Dickens, 1932, Armstrong and Dickens, 1935, Allen and Spencer, 1935, Collis, 1935, Birch, 1936, Burgers, 1936, McLean, 1936, Riesman, 1936, Hughes, 1937, Sreenivasan, 1940, Jennings, 1947) Useful general reviews on lymphocytic choriomeningitis have been published by Kreis (1937) and Farmer and Janeway (1942)

FEATURES OF HUMAN INFECTION WITH LCM VIRUS

The incubation period is probably from 1 to 3 weeks Prodromal symptoms are common, may last 7–20 days, and include remittent fever, chills, influenza-like symptoms, sore throat and tonsillitis, cough, and bronchitis (Farmer and Janeway, 1942) Then the typical meningeal symptoms develop, with headache, vomiting, stiffness of the neck, and photophobia The course is usually short and the majority of cases recovers in a few weeks In some instances the infection may recrudesce, even up to a total of

It is now known rm of illness, other syndromes may 1942; Milzer, 1943) In some cases ental changes, aphasia, diplopia, and paralysis (Findlay, Alcock, and Stern, 1936, Farmer and Janeway, 1942) In other cases, the illness is of an influenzal nature with viremia, but without clinical evidence of localization of virus in the CNS (Armstrong and Hornibrook, 1941). Smadel *et al* (1942) described a case of apparent virus pneumonia.

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(Milzer and Levinson, 1942; Smadel *et al.*, 1942; Yamarat, see Farmer and Janeway, 1942; Hayes and Hartman, 1943). The route of infection in these patients has been obscure, but probably the conjunctiva, respiratory tract, or skin has been the portal of entry

Clinical pathology. Leukopenia is commonly observed in the early stages, and this is followed by polynucleosis. The cerebrospinal fluid shows an increase in lymphocytes up to 3,000 per c mm The protein is slightly increased, but the sugar and chloride are normal

Distribution of the virus. In all types of infection, the virus is probably present in the blood in the early stages Later it invades the central nervous system, and is present in the CSF for the first 7–10 days of meningeal symptoms; it has been recovered in a relapsing case 13 weeks after the initial attack (Leichenger, Milzer, and Lack, 1940) The virus has been recovered from the CSF, even in the absence of definite pleocytosis (Milzer and Levinson, 1942, Treusch, Milzer, and Levinson, 1943). This virus may also be found in the nasopharynx (MacCallum and Findlay, 1939), and urine (Lépine and Sautter, 1938).

The pathological features have been described by various authors (Viets and Warren, 1937, Machella,
1940; Mitchell and Klotz,
cells, and macrophages.
inflamed Changes in the brain, and sometimes cord, include perivascular lymphocytic cuffing, hemorrhages, gliosis, and degeneration of nerve cells In one case chronic arachnoiditis developed and resulted in spastic paraplegia (Barker and Ford, 1937).

Laboratory diagnosis. Some cerebrospinal fluid (not over 0 03 c.c) should be injected intracerebrally in mice Precautions should be taken to insure that the mouse stock used is itself virus-free. Guinea-pigs should also be injected (intracerebrally or subcutaneously) The spleen of guinea-pigs or mice may be tested as early as 5–6 days after inoculation for fixation of complement with hyperimmune serum (Smadel and Wall, 1941). If available, a macaque may be injected cerebrally. Finally, the convalescent serum may be tested for the development of virus neutralizing and complement fixing antibodies, about 12 weeks after onset.

LYMPHOCYTIC CHORIOMENINGITIS IN MICE AND OTHER ANIMALS

Laboratory stocks of apparently healthy mice in all parts of the globe *are liable to* suffer from natural infection, and the presence of LCM virus must always be excluded when any new viral agent is isolated in mice, further, the virus may be passed in series
iboratory contaminant (Traub, 1935 *a*, *b*,
and Sautter, 1936) Mortality in infected
inimals show no symptoms, although the
sal secretions Many animals are resistant
s developing evidence of infection, such
ig, from 2–6 weeks old The intracerebral
inoculation of broth in infected animals induces the development of meningitis Histologically, naturally infected animals show mild meningeal infiltrations and focal necroses in the liver Traub (1939), examining a stock of mice infected with the virus for 4 years, found the infection to have become symptomless, infection took place by intra-uterine spread, and *not by contact*

LCM virus has been encountered in other animals Thus Dalldorf (1939 *b*) found that a strain of canine distemper passed in dogs and ferrets had become contaminated It has been encountered in monkeys (Armstrong and Wooley, 1935, Coggeshall, 1939) The virus appeared as a contaminant in cultures of rabies virus, where mouse brain and monkey serum were used (Casals-Ariet and Webster, 1940)

EPIDEMIOLOGY

Small outbreaks of aseptic meningitis have been described (Laubry and Foy, 1910, Rist and Rolland, 1910, Wallgren, 1925, Eckstein, 1931, Dummer, Lyon, and

Stevenson, 1937). It is very unlikely, however, that these outbreaks were associated with the LCM virus, for infections with this agent are invariably sporadic, and case to case transmission is virtually unknown

The disease occurs in all parts of the world.

The Routes and Sources of Infection

The route of infection is not definitely known. It is certain, however, that the virus can enter the body by the conjunctiva (Lépine and Sautter, 1938). It can probably also pass through the skin, or be inhaled in the form of infected droplets or dust particles (Findlay, Alcock, and Stern, 1936) Cases have been recorded following tonsillitis (Riesman, 1936). It has been suggested that the infection may be acquired venereally (Wooley, Armstrong, and Onstott, 1937).

Case to case infection is extremely rare, the possibility of human carriers infecting by means of droplets is remote.

Main suspicion falls on *mice* as the source of infection. As mentioned above, laboratory mice are very liable to be infected with the virus. Observations have shown that a considerable percentage of house mice, perhaps up to 20 per cent., caught in random samples in urban areas carry the virus, or prove resistant to challenge, embryos have been found infected (Armstrong, Wallace, and Ross, 1940, Yamarat, see Farmer and Janeway, 1942, Dalldorf, Jungeblut, and Umphlet, 1946). Probably mice caught in rural areas may also be infected (Howitt and van Herick, 1942). Further, the virus has been isolated from mice trapped in the homes of cases of the disease (Armstrong and Sweet, 1939, Armstrong, Wallace, and Ross, 1940). Dalldorf, Jungeblut, and Umphlet (1946) reported a small focus of 4 cases in close neighbors, and isolated virus from mice trapped in the dwelling.

Another point suggesting the rôle played by mice is the tendency for the greater incidence of cases in spring when the mice are breeding, and again in the autumn when colder weather drives them indoors (Armstrong, 1941).

The method of transmission of virus from mouse to man is probably by inhalation of dried urine A case has been reported where infection may have arisen through handling a dead infected mouse (Farmer and Janeway, 1942)

Mosquitoes have been incriminated, and on an analogy with virus encephalitis, might be expected to play some part. The evidence is, however, experimental rather than epidemiological Coggeshall (1939) showed that *Aedes* could transmit the infection to guinea-pigs as early as the 4th, and at least as late as the 15th day after feeding on an infected animal Milzer (1942) obtained similar results, when *Aedes* were incubated at temperatures between 26 and 37° C transmission was obtained from 7–38 days after the infective meal

Bedbugs can also transmit the infection to guinea-pigs, partly by bite, and partly by the rubbing in of infected feces (Milzer, 1942)

Lice have also been blamed The virus is retained for at least some hours in the human body louse fed on the infected monkey (Findlay, Stuart-Harris, and MacCallum, 1940) The monkey louse can transmit the infection to monkeys (Armstrong, 1941), and probably man (Milzer and Levinson, 1942)

The tick (Dermacentor andersoni) has been infected at all stages, and adult to egg transmission occurs, infection was transmitted to guinea-pigs by feeding nymphs which had engorged in their larval stage on an infected animal, infection was also transmitted by applying infected crushed ticks or tick feces to the scarified skin (Shaughnessy and Milzer, 1939).

The nematode Trichinella spiralis has been incriminated experimentally (Syverton, McCoy, and Koomen, 1947) The larvae after maturation in the muscles of infected guinea-pigs acquired the virus and could transmit it to new hosts, transmission resulted both when living larvae were fed to normal pigs and when triturated dead larvae were injected subcutaneously

(Milzer and Levinson, 1942, Smadel *et al.*, 1942, Yamarat, see Farmer and Janeway, 1942; Hayes and Hartman, 1943). The route of infection in these patients has been obscure, but probably the conjunctiva, respiratory tract, or skin has been the portal of entry.

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The pathological features have been described by various authors (Viets and Warren, 1937, Machella, Weinberger, and Lippincott, *1939*, Silcott and Neubuerger, 1940, Mitchell and Klotz, 1942). The meninges are infiltrated with lymphocytes, red cells, and macrophages The choroid plexuses and ventricular ependyma may be inflamed. Changes in the brain, and sometimes cord, include perivascular lymphocytic cuffing, hemorrhages, gliosis, and degeneration of nerve cells. In one case chronic arachnoiditis developed and resulted in spastic paraplegia (Barker and Ford, 1937).

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Laboratory stocks of apparently healthy mice in all parts of the globe are liable to suffer from natural infection, and the presence of LCM virus must always be excluded when any new viral agent is isolated in mice, further, the virus may be passed in series unknowingly along with another agent, as a laboratory contaminant (Traub, 1935 *a*, *b*, 1936 *a*, *b*, Findlay,
mouse stocks is le
virus is present in
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as emaciation, drowsiness, and ruffled fur, are young, from 2–6 weeks old The intracerebral inoculation of broth in infected animals induces the development of meningitis Histologically, naturally infected animals show mild meningeal infiltrations and focal necroses in the liver Traub (1939), examining a stock of mice infected with the virus for 4 years, found the infection to have become symptomless, infection took place by intra-uterine spread, and not by contact

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EPIDEMIOLOGY

Small outbreaks of aseptic meningitis have been described (Laubry and Foy, 1910, Rist and Rolland, 1910, Wallgren, 1925, Eckstein, 1931, Dummer, Lyon, and

necrosis of liver, thymus, spleen, lymph nodes, and marrow, thrombi in liver and other viscera, and lymphocytic infiltrations in the pleura, peritoneum, renal cortex and pelvis, liver, salivary glands, pancreas, thoracic organs, and adrenals

Traub (1938 *b*) has reported that mice may become infected *in utero*, or during suckling, animals infected *in utero* carried virus in the blood longer than those infected during suckling. In mice infected during suckling or after intranasal instillation, it was the younger ones that remained carriers for the longer time Haas (1941) found that mice infected *in utero* or early infancy retained active virus for long periods, and transmitted infection to offspring and contacts Mice infected when mature did not transmit infection to offspring, except when the animals were pregnant at the time of inoculation.

Rabbits.

Rabbits show no symptoms after introduction of virus by the usual routes, but become actively immune (Findlay and Stern, 1936, Traub, 1936 *a*, Rivers and Scott, 1936).

Guinea-pigs.

These animals can be infected readily by cerebral or peripheral routes (Findlay and Stern, 1936; Rivers and Scott, 1936, Scott and Rivers, 1936, Traub, 1936 *a*, *b*, Lépine, Kreis, and Sautter, 1937, Ronse, 1937, Mendoza, 1937) Viremia develops 24 hours after inoculation, and persists till death (Shaughnessy and Milzer, 1939, Milzer, 1942). The virus is firmly bound to the red cells (Shwartzman, 1943, 1946)

The pathological features have been fully described by Lillie and Armstrong (1944): There is a relatively mild and chiefly basilar lymphocytic meningitis and focal lymphocytic infiltration of the choroid plexus. Foci of lymphocytic infiltration occur in the heart and lungs, and perhaps other organs Focal necrotic and proliferative lesions occur in the liver. The spleen shows an early polymorph infiltration, with later a reticulo-endotheliosis and lymphoid cell infiltration. Follicle hyperplasia occurs in the spleen, lymph nodes, and intestinal lymphoid tissue

After intracerebral injection, the majority of animals dies from a mild meningitis with "virus" pneumonia. After serial passage through mice, Traub (1937) attenuated a strain so that, on intracerebral injection, it produced only fever.

After subcutaneous and, less commonly, after intraperitoneal injection fever develops, the animal salivates, becomes emaciated, and finally dies in 9 to 16 days Virus may be found in the blood and hemopoietic system, brain, urine, and lungs. It persists in the urine for some weeks after recovery, but not in the blood (Traub, 1936 *b*) Infection can also take place through scarified or even unbroken skin (Shaughnessy and Milzer, 1939, Shaughnessy and Zichis, 1939, 1940).

Other rodents.

After intracerebral injection, rats may die from choriomeningitis, but this is uncommon After intraperitoneal and other peripheral injections no symptoms are manifest, although antibodies may develop and the virus be recoverable from the blood, brain, liver, spleen, and urine (Findlay and Stern, 1936, Traub, 1936 *a*, Lépine, Kreis, and Sautter, 1937). *Sigmodon* and *Microtus* can be infected (Howitt and van Herick, 1941), also Syrian hamsters (Smadel and Wall, 1942).

Monkeys.

N[illegible]

L[illegible]

a[illegible]

A[illegible]

The temperature rises 4 to 8 days after intracerebral injection and remains up (104° F.) for 3 to 10 days, the animal goes off its food, loses weight, is very quiet

PROPERTIES OF THE VIRUS

There is no evidence that different antigenic types exist (see, e g, Milzer and Levinson, 1942).

Human Inoculation Experiments

Lépine, Mollaret, and Kreis (1937) inoculated human beings subcutaneously. Fever of an undulant type developed after 36 to 48 hours and lasted for up to 3 weeks; the clinical features resembled influenza. About half the patients showed a definite meningeal reaction with abrupt onset of headache, vomiting, and Kernig's sign. The virus was demonstrated in the blood and cerebrospinal fluid.

Animal Experiments

Guinea-pigs and mice are chiefly used for the isolation of virus and its passage in the laboratory. Shwartzman (1946) studied the viscero- and neuro-tropic properties of substrains passed in series through guinea-pigs and mice All substrains were neurotropic for the mouse, irrespective of the animal used for previous passage. Viscerotropism was acquired by serial passage through the guinea-pig, and lost on serial passage through the mouse.

Mice.

Mice can be infected readily (Armstrong and Lillie, 1934, Armstrong and Wooley, 1935; Rivers and Scott, 1936, Findlay, Alcock, and Stern, 1936, Findlay and Stern, 1936, Lépine and Sautter, 1936, Traub, 1936 *a*, *b*, Lépine, Kreis, and Sautter, 1937, Ronse, 1937). Young mice are more susceptible than older animals, especially to peritoneal inoculation (Casals-Ariet and Webster, 1940). Young mice tend to retain virus for life, whereas it is difficult to demonstrate virus in older mice, except shortly after inoculation (Haas, 1941). Viremia commonly develops 24 hours after inoculation, and persists for at least 7 days, following cerebral, subcutaneous, or peritoneal inoculation (Milzer, 1942) Strains of high virulence for the mouse show a firm association of virus with the red cells (Shwartzman, 1943, 1944, 1946) The pathology has been fully described by Lillie and Armstrong (1945)

Intracerebral injection. Mice show evidence of infection on the 6th to 7th day, the fur is ruffled, there are coarse tremors of the head, and if the animal is picked up by the tail, it makes very rapid convulsive movements, which last for a matter of seconds or up to a minute. In the later stages these convulsions occur frequently without outside stimulation. The animal usually dies in 1 to 3 days.

Histologically, the brain shows an abundant lymphocytic infiltration of the meninges with occasional hemorrhages most marked basally The plexuses show abundant exudate and there is some glial proliferation of the walls of the lateral ventricles The ventricles themselves are filled with lymphocytes, plasma cells, and some polymorphs. There is little nerve cell destruction, and perivascular cuffing is only marked near the ventricles

Focal necrotic lesions are found in the liver and spleen Apart from the brain the virus is to be found in whole blood, plasma, urine, spleen, liver, lungs, kidneys, and adrenals

Nasal instillation Encephalitis develops after nasal instillation

Other routes of infection An indefinite nonfatal illness with development of immunity follows the introduction of virus by the following routes scarification of skin, subcutaneous, intraperitoneal, intravenous, and instillation of the external ear. Contact infection can occur Even after peripheral inoculation, the brain may show changes, visceral lesions, infrequent after cerebral inoculation, are more pronounced when other routes are used (Lillie and Armstrong, 1945). The following changes have been observed polyserositis, fatty degeneration of liver and kidneys, focal

1941), or an extract irradiated with UVL (Casals, 1942; Havens *et al.*, 1943). Complement fixing antisera can be prepared readily in hyperimmune guinea-pigs.

Complement fixation depends partly on the elementary body, but more particularly on a soluble substance (Smadel, Baird, and Wall, 1939 *a*, *b*). The properties of this soluble substance are as follows (1) SS can be separated from virus, by centrifugation and Seitz filtration, washed virus fixes complement poorly, SS is widely distributed in the tissues, especially the spleen and brain, of infected guinea-pigs, mice, and monkeys (Smadel, Baird, and Wall, 1939 *b*) (2) SS is relatively stable, and resists 56° C. for ½ hour, it is of protein nature (Smadel, Wall, and Baird, 1940). (3) Precipitation occurs when serum is added to solutions of antigen (Smadel, Wall, and Baird, 1940). (4) SS can be kept in solution in the refrigerator for months, or it can be stored if dried from the frozen state (Smadel and Wall, 1941) It resists irradiation (Havens *et al*, 1943) (5) Formalin-treated virus-free extracts, containing large amounts of SS, fail to elicit antibodies to SS (or to induce resistance) in normal guinea-pigs, when administered to immune guinea-pigs, however, there is a marked increase in the titer of anti-SS substance. On the other hand, suspensions of formolized washed virus are effective in normal guinea-pigs in stimulating the development of both anti-SS and neutralizing substances, and inducing resistance (Smadel and Wall, 1940)

Virus neutralization has also been studied, and hyperimmune animal or convalescent human sera neutralize the infectivity of virus, serum-virus mixtures are usually inoculated cerebrally in mice or subcutaneously in guinea-pigs (Armstrong ... 'ey, 1935; Lépine and Sautter, 1936; Findlay, ... :ott, 1936, Scott and Rivers, 1936; Traub, ... Armstrong, and Onstott, 1937, Howard, 1939, ... 1939, ... and Levinson, 1942).

The interrelationship of the various antigen-antibody reactions is as follows Precipitation and complement fixation are dependent on the interaction of SS with the same antibody, but the neutralizing antibody appears to be separate (Smadel and Wall, 1940, Smadel, Wall, and Baird, 1940).

Active Immunity

Guinea-pigs can be rendered partly resistant by injection of formolized virus (Traub, 1938 *a*, Smadel and Wall, 1940).

Mice can be rendered resistant by peripheral injection of live virus (Traub, 1938 *b*, Casals-Ariet and Webster, 1940, Lyon, 1940)

Antibody Response in Man

nor ... ma ... bee ... Milzer, and Levinson, 1943).

On recovery from infection, neutralizing antibodies develop rather slowly, and have been detected at 8 weeks from onset, the titer increases to a maximum about 6 months after onset, and reaches a constant level after 12 months, which is maintained for a considerable period (Farmer and Janeway, 1942, Milzer and Levinson, 1942)

C ... r, and have been detected 3–5 ... at least 2 years (Farmer and ...

RELATIONSHIP TO OTHER VIRUSES

There is no relationship between LCM and the following viruses influenza or LGI virus (Traub, 1936 *a*), Durand's virus (Findlay, 1942), Japanese, St Louis, West Nile, western or eastern equine encephalomyelitis viruses (Howitt, 1936–7,

and apathetic, and may show intention tremors of the hands; death may result, but certain animals recover and show active immunity. The cerebrospinal fluid shows a lymphocytosis and the blood a polynucleosis. Monkeys can also be infected *intraperitoneally*, but *encephalitis does not develop*. *Armstrong, Wooley, and* Onstott (1936) found virus in brain, spinal cord, and cerebrospinal fluid, blood, adrenals, kidneys, liver, lungs, lymph nodes, marrow, heart, voluntary muscle, pancreas, spleen, and testicles. Lillie (*1936 b*) reports the histological features in infected monkeys as follows. lymphocytic infiltration of the plexuses and meninges, some perivascular infiltration in brain and cord, focal lymphocytic infiltration in root ganglia, congestion and interstitial edema of the lungs; pyelitis and hemorrhagic cystitis; focal hepatic necrosis, and lymphocytic infiltration of the heart, testis, muscles, and abdominal organs.

Insusceptible animals.

Chicks, pigeons, dogs, ferrets, hedgehogs, voles, hens, canaries, and parakeets show no symptoms (Traub, *1936 a*; Findlay, Alcock, and Stern, *1936*).

Chemotherapy

Prontosil was stated to have a protective effect against infection in mice when injections were begun early in the incubation period (Rosenthal, Wooley, and Bauer, 1937), other authors, however, have not confirmed these findings (see MacCallum and Findlay, 1938, McKinley, Meck, and Acree, 1939, Toomey and Takacs, 1944)

Morphology and Cultivation

The virus probably measures between 33 and 60 mμ, as determined by filtration or centrifugation (Rivers and Scott, *1936*, Scott and Elford, *1939*, Casals-Ariet and Webster, *1940*) It passes the following filters Berkefeld V, N, and W, Chamberland L_1, L_2, L_3, Seitz EK (Armstrong and Lillie, *1934*; Rivers and Scott, *1936*, Findlay, Alcock, and Stern, *1936*; Lépine, Kreis, and Sautter, *1937*).

The virus can be cultivated on the chorio-allantois (*Bengtson and Wooley*, *1936*, Tubaki, *1940*, Milzer and Levinson, *1942*). However, no characteristic pocks or other lesions develop, and there are no specific lesions (Lillie, *1936 a*)

It can be cultivated in minced chick or mouse embryo, serum, and Tyrode (MacCallum and Findlay, *1940*, Casals-Ariet and Webster, *1940*)

Reaction to Physical and Chemical Agents

The virus resists 50 per cent. neutral glycerol for at least 7 months. It maintains its virulence at 5° C in a suspension under a vaseline seal for at least 2 months, and in a Petri dish for 1 month After desiccation from the frozen state it remains active for at least 12 months The virus is destroyed by 55° C in 20 minutes (Armstrong and Lillie, 1934, Findlay, Alcock, and Stern, 1936, Rivers and Scott, 1936, Lépine, Sautter, and Kreis, 1937, Wooley, 1939) It is rapidly inactivated by UVL (Havens *et al*, 1943) It is inactivated by various fatty acids and detergents (Stock and Francis, 1943).

IMMUNITY

Antigen-Antibody Reactions

Complement fixation can be demonstrated readily between suspensions of infected tissues and animal or human hyperimmune or convalescent serum (Howitt, 1936–7, Lépine and Sautter, 1938, Lépine, Mollaret, and Sautter, 1938, Milzer and Levinson, 1942).

Various types of antigen can be used e g, a saline extract of infected spleen, an extract of infected brain prepared by freezing and thawing (Casals and Palacios,

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1937; Milzer and Levinson, 1942; Havens *et al*, 1943); feline agranulocytosis virus (Lawrence *et al.*, 1943); EKC virus (Sanders and Alexander, 1943). Dalldorf (1939 *a*) detected a sparing effect, partly reciprocal, between LCM, and poliomyelitis in monkeys.

THE VIRUS OF PSEUDOLYMPHOCYTIC CHORIOMENINGITIS

MacCallum, Findlay, and Scott (1939) gave this name to a virus isolated on two occasions from the cerebrospinal fluid of patients suffering from benign aseptic lymphocytic meningitis. It resembles LCM closely, except that in mice the incubation period is only 4 to 5 days, and the lesions are less extensive.

SWINEHERD'S DISEASE

This disease is also known as *maladie des porchers*, or eruptive meningotyphoid, and is characterized by a febrile period with gastro-intestinal disorder and a rash, followed by a remission, after which meningeal signs develop (see Penso, 1936). It occurs chiefly in France, Italy, and Switzerland in swineherds and others who come into close contact with sick pigs Infection appears to be contracted from these animals, perhaps by the excreta, and not from human cases. The infection appears to be transmissible to man by inoculation of blood (Durand *et al*, 1937, Georgi, Pache, and Urech, 1938) Claims have been made that the causal agent is a filtrable virus transmissible to animals (*e.g*, Durand *et al*, 1936, Penso and Rosa, 1938), but insufficient work has yet been carried out.

HUMPHREYS' VIRUS

This strain was isolated in guinea-pigs inoculated with a suspension of ticks (Humphreys, Helmer, and Gibbons, 1944) It was highly pathogenic for guinea-pigs, and grew in the chick embryo. The histological lesions show less necrosis, edema, and fibrin, and more lymphocytic infiltration, but otherwise correspond to those obtained in LCM infection (Lillie and Armstrong, 1945). Perrin and Steinhaus (1944) also remarked on histological differences. It does not appear to be possible on the evidence available to decide whether this virus is identical with LCM, but it is certainly closely related

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CHAPTER XC

DURAND'S VIRUS

This was isolated by Durand (1940) from his blood during a febrile illness in 1939 The main symptoms were fever, constipation, and insomnia The illness lasted 11 days, and was followed by asthenia before complete recovery. A virus known as "D" was isolated by intraperitoneal inoculation in guinea-pigs, and the strain was passed in series. D virus has also been studied by Findlay (1942).

Infection in Man

Experimental inoculation produces a local reaction, headache, fever, and viremia, the CSF may contain virus (Durand).

Findlay described a laboratory infection characterized by nasal catarrh, dry cough, lethargy, fever, headache, vomiting, and loss of weight. Virus was isolated from the blood

Infection in Animals

Guinea-pigs can be infected by the peritoneal, subcutaneous, cutaneous, muscular, testicular, nasal, cerebral, and ocular routes (Durand, Findlay). The most characteristic lesions are found following subcutaneous injection. The incubation period varies from 3–10 days, largely according to the amount of virus inoculated Illness is characterized by fever, lasting 6–10 days There is a local inflammatory reaction at the site of inoculation, with regional adenitis The animal is generally "off-color," and develops conjunctivitis and dyspnea Loss of weight is considerable Animals usually recover, but death may occur from rupture of the spleen, or generalized lesions. Autopsy shows evidence of loss of weight, there is hemorrhagic edema at the site of inoculation, with lymphadenitis, the spleen is enlarged, the lungs are congested and show interstitial pneumonia (Durand, Findlay). The in-

Properties of the Virus

The virus passes a Chamberland L_2 candle, a Seitz EK pad, and all grades of Berkefeld filter, the size, determined by filtration, is between 53 and 80 mμ, it can be kept in the ice chest for some weeks, it can be preserved in glycerine, or by freezing and drying (Durand, Findlay).

Findlay cultivated the virus in chick embryo tissue in serum and Tyrode; he also found that virus generalized in the egg and might cause death after yolk sac or chorio-allantoic inoculation

Immunity

Recovered guinea-pigs are immune to reinfection from 3 weeks after the end of the febrile period, and this resistance lasts for several months, immunity can be induced with killed virus (Durand, Findlay)

In convalescence, virus-neutralizing antibodies develop in man, rabbits, guinea-pigs, and other animals, complement fixation can also be demonstrated (Durand; Findlay).

Relationship to Other Viruses

Findlay found no evidence of cross immunity between virus D and LGI or LCM

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myelitis is the primary manifestation of infection, and which will be discussed in the following pages The causal agents of encephalitis lethargica and Australian X disease have not been isolated, but are presumed to be viruses. Encephalitis can be produced by the following neurotropic viruses St Louis, Japanese, Russian, North and South American equine encephalomyelitis, and louping ill Certain viruses, e g, the West Nile virus, isolated by yellow fever workers (see Section 4) are also neurotropic in animals, but at present we do not feel that enough is known of their pathology in man to justify their classification in this section

For the sake of completeness we should mention that the specific neurotropic infections poliomyelitis and rabies have already been discussed in special sections, and so have the infections characterized by meningitis rather than encephalitis

Finally, various outbreaks and sporadic cases of encephalitis have been described, where it appears highly likely that a virus is concerned. For various reasons, however, no special virus studies have been made. Such cases have occurred in the following areas: in South Central Africa and Kenya (Charters, 1940, Berney and Gelfand, 1946); in the Argentine (Valdes, 1943); in troops in N. Australia, Horan *et al.* (1944) described 2 fatal cases due to an unknown agent entering peripherally, spreading by nerve paths, and producing focal necroses of the nervous tissues and of the walls of small and medium veins, in Brazil (Di Lascio, 1943), in Germany (Gildemeister and Haagen, 1940), in India (Chatterji, Gupta, and De, 1945); in Sweden (Moller, 1939), and in Texas (Woodland and Smith, 1942).

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SECTION 11. DISEASES CHARACTERIZED CHIEFLY BY ENCEPHALITIS, ENCEPHALOMYELITIS, OR MYELITIS

CHAPTER XCI

THE ENCEPHALITIS GROUP OF VIRUSES: INTRODUCTORY

ENCEPHALITIS and encephalomyelitis occur in various circumstances, and although we are concerned only with virus infections, it is desirable first to mention some other types of known as well as of uncertain etiology.

For example, encep[illegible] meningo-cocci, and may occur [illegible] the exhibition of ent[illegible] amides (Brain, 1943).

Histological changes similar to those seen in infective encephalitis may be produced by carbon monoxide poisoning, brain injury, arteriosclerosis, uremia, pregnancy toxemia, and toxic agents like alcohol and lead (Toomey, 1940, Putnam, 1941)

Although we cannot enter into the vexed question of the etiology of disseminated sclerosis, it is of interest to note that it has been claimed recently that some cases may be of virus origin (Margulis, Soloviev, and Shubladze, 1946). Further, it has been reported that disseminated sclerosis developed in 4 out of 7 workers studying the disease of lambs known as sway-back (Campbell *et al.*, 1947), this disease is associated with demyelination. There has hitherto been no definite evidence that sway-back is due to a virus, and copper deficiency appears to be of importance. These new observations, however, show that the question requires fresh consideration

Another interesting disease of uncertain etiology is known as "acute hemorrhagic leuko-encephalitis" (Hurst, 1941, Henson and Russell, 1942, Greenfield, 1943, Russell, 1943, Shallard and Latham, 1945). It is characterized by rapid loss of higher functions, and histologically by severe damage to the walls of blood vessels, perivascular necrosis, focal and perivascular demyelination, marked polymorph infiltration, microgliosis, edema, and hemorrhages.

Encephalitis may be produced by the causal agents, probably viruses, of glandular fever and infectious hepatitis (Thelander and Shaw, 1941, Brain, 1943)

A type of encephalitis that may be due to a virus is that known as "inclusion" encephalitis There are acidophilic intranuclear inclusions like those of herpes in the ganglionic cells of the cortex (Brain, 1943, Greenfield, 1943, Russell, 1943)

A disease from which a virus has been isolated is that known as "acute primary [illegible] encephalitis" occurring in Russia and other parts of Europe [illegible] 1944)

[illegible] virus infections, encephalitis and encephalomyelitis may occur as complications of the following, most of which are fully discussed elsewhere in this book variola, varicella, mumps, rubella, measles, influenza, *herpes febrilis*, herpes zoster, as well as rickettsial diseases Encephalomyelitis may follow the inoculation of rabies vaccine or vaccinial lymph Perivascular demyelination is a characteristic finding in many of these conditions

There is also a large number of virus diseases where encephalitis or encephalo-

mentioned, these contain nearly every reference to published work and should be consulted for fuller information than can be given in this chapter.

Clinical Features

The incubation period varied from 3 to 21 days, the usual interval being 10 to 14 (Parsons, MacNalty, and Perdrau, 1922). *Prodromal symptoms* of lethargy, insomnia, headache, vertigo, dry mouth, constipation, hiccup, dysuria, tremors, diplopia, tinnitus, and severe pains might occur; the temperature often rose, to fall in the course of a few days (Bramwell, 1921). *The onset* of the disease proper occurred in one of four ways (*a*) asthenic or somnolent type; (*b*) the meningitic type, (*c*) apoplectiform type, (*d*) with neuralgia referred to one or more extremities, or the head (Bramwell and Miller, 1920). MacNalty (1918) described the manifestations of the established disease as follows

General symptoms Conjunctivitis, headache, vomiting, pyrexia, sweating, skin eruptions, and edema might all occur. These symptoms were usually [illegible] with one or more features indicative [illegible]

centr[illegible]

ters [illegible]

oph[illegible] [illegible], strabismus, diplopia, and bilat[illegible] [illegible] palsies were all observed The 7th nerve was sometimes affected, giving a bilateral facial paralysis. The various motor nuclei of the lower cranial nerves might be attacked, giving, for instance, aphonia The face was characteristically impassive and mask-like

Certain cases of acute febrile chorea were described (particularly in France) which were true cases of encephalitis lethargica (see Brouardel, Levaditi, and Forestier, 1920, Levaditi and Harvier, 1920 *e*) Epidemics of hiccup were observed in various parts of the world (see Boyd, 1920 *a*, *b*, Parsons, MacNalty, and Perdrau, 1922), coexistent with the epidemic of encephalitis [illegible]

was due to a [illegible]

In childre[illegible]

palsy de[illegible]

were c[illegible]

Wal[illegible] [illegible] of encephalitis had a complex action on the central nervous system Either its action was a paralyzing one, affecting only the basal ganglia or brain stem, or it was irritant, when it affected any and every part of the central nervous system This theory certainly explains the varied nature of the nervous manifestations

Clinical Pathology

Acute stage The [illegible] authors (see, e g, Netter, 1920 *a*, [illegible], and Perdrau, 1922). The cell coun[illegible] [illegible] in proportion to the severity of the infection and although seldom more than 20 to 30 cells per c mm. were found, counts up to 90 were recorded on occasions. In almost every case the increase was due to a lymphocytosis Sugar was occasionally increased up to 0 1 per cent (Netter, 1920 *a*, Foster, 1921) Total protein showed a small increase in many cases (Parsons, MacNalty, and Perdrau, 1922) Colloidal gold curves were frequently luetic and less commonly paretic in type (Findlay and Shiskin, 1921, see also Douglas, 1926)

Chronic stage According to Parsons, MacNalty, and Perdrau, the cerebrospinal fluid was normal except for slight inconstant increases in the number of lymphocytes, total protein, and chloride content

CHAPTER XCII

ENCEPHALITIS LETHARGICA

Historical

In the 16th, 17th, 18th, and 19th centuries there were numerous outbreaks in Europe of a disease characterized by somnolence So pronounced a feature was this, that the disease became known as sleepy sickness, *Schlafkrankheit*, or *nona*, according to the country affected. Full accounts of these early outbreaks, which were probably due to encephalitis lethargica, are to be found in various papers (Netter, 1920 *a*, *b*, Zinsser, 1928, Abrahamson, 1935).

Modern interest in encephalitis lethargica only dates from World War I, when the disease was first described independently in France by Cruchet, Montier, and Calmettes (1917), and in Austria by von Economo (1917). The main clinical features of these outbreaks were somnolence, oculomotor palsies, signs of cerebral and meningeal irritation, and pyrexia. It was recognized at once that this disease was to all intents new

[illegible]

due to a cultivable organism

The disease soon assumed pandemic distribution and the first cases in England were recorded by Harris (1918) and Hall (1918 *a*, *b*) At this early stage the features were those of drowsiness, pyrexia, and ophthalmoplegia, and therefore the question of botulism naturally arose, especially in view of the nature of the foodstuffs available toward the end of the war. It was soon found, however, that the disease was not due to any form of food poisoning but was a specific entity (*Rep. loc. Govt Board*, N S 121, 1918)

Netter (1918, see also 1920 *a*, 1921 *a*, *b*) described some of the early cases in France. The disease appeared in the Far East in 1919 and spread rapidly (see Pfister, 1929). The early cases in America were reviewed by Bassoe (1919) and Smith (1921). In Canada, Boyd (1920 *a*, *b*) described an outbreak in Winnipeg By 1920 the disease had assumed a world-wide distribution

FEATURES OF ENCEPHALITIS LETHARGICA AS SEEN FROM 1918 TO 1920

Incidence of the Disease in the British Isles

The disease was made compulsorily notifiable as from January 1, 1919, in England and Wales, and in 1919 the number of cases thus reported was 541 In 1921 this figure had risen to 1,470 A temporary abatement occurred in 1922 (454 cases), but the disease broke out again in 1923 (1,025 cases), and assumed its most serious proportions in 1924 (5,039 cases) From that time onward the numbers of notifications have gradually fallen as follows 1925—2,635 cases, 1928—1,308 cases, 1931—651 cases, 1934—411 cases, 1936—269 cases, 1937—217 cases

The disease was made the subject of a number of official reports, the main findings of which are described later (*Rep loc Govt Board*, N S. 121, 1918, *Suppl 48th Ann Rep. loc Govt Board*, London, 1919, *Ann Rep. Chief M O Min Hlth*, Append. 7, London, 1919–20, *Rep publ Hlth med Subj*, no 11, London, 1922, *Med res Counc spec Rep*, no 108, London, 1926)

In addition, the surveys of the Matheson Commission (1929, 1932) should be

temporary incapacity months before. The general symptoms are usually restricted to those of pyrexia. Nervous manifestations are varied, and include paralyses of the cranial nerves (especially ophthalmoplegias), irritative twitchings, oculogyric crises, and choreiform movements. Lethargy is usually found only in cases showing paralysis

At the same time it must be realized that the disease may present itself in any of the "types" already described.

It has been estimated that approximately one-third of patients recover from the acute attack only to develop characteristic sequelae of which parkinsonism is the commonest

According to Lapage (1933) the main features of encephalitis lethargica in childhood are as follows Lethargy is commonly found, deepening into coma in severe cases Headache is common and very persistent. Although diplopia may be present the child may not actually complain. Hiccup is a common symptom, and myoclonic and paralytic cases may occur. Sequelae are common and take the form of [illegible] the [illegible] the

PATHOLOGY

A. Acute Infections of the Central Nervous System

Naked-eye, there is frequently a diffuse pinkness of the brain, and on section small pin point hemorrhages may be found in the gray matter. There is not, however, any particularly diagnostic feature and, in fact, the brain often appears quite normal to the naked eye.

Microscopically, Marinesco (1918 *a*, *b*) reported on some of the first cases of the disease that occurred in Great Britain, numerous others have also published descriptions (e g, von Economo, 1917, von Wiesner, 1917, Marie and Trétiakoff, 1918, Boyd, 1920 *a*, *b*, Bramwell and Miller, 1920, Levaditi and Harvier, 1920 *e*, MacNalty, 1921, Hume, Nattrass, and Sh[illegible].

The characteristic lesions are usually [illegible] ing regions, and are most marked in th[illegible] aqueduct of Sylvius, 3rd ventricle, posterior part of pons and peduncles, and to a limited extent in the upper part of the cord These lesions are of a number of types

1 Infiltration of the adventitial coats of the smaller vessels (especially venules) with lymphocytes and plasma cells. In the more acute cases there are large numbers of polymorphs to be found, even constituting miliary abscesses (Hume, Nattrass, and Shaw, 1922)

2. Foci of interstitial inflammation, consisting of neuroglia with large excentric nuclei and sometimes fibrillary projections, are present

3 Lesions of the nerve cells take the form of lysis of the Nissl bodies, satellitosis, *and reduction in the volume of the cell body* and the number of its processes There is a notable diminution in the number of nerve cells in the dorsal nucleus of the vagus, locus ceruleus, and locus niger. Neuronophagia is not a frequent finding

4 Foci of hemorrhage are found around the smaller vessels, particularly near the sylvian aqueduct Many vessels contain large numbers of polynuclears, almost constituting a thrombus Less frequently hemorrhages may be found in the cerebral substance

5 Parenchymatous infiltrations with lymphocytes, polymorphs, plasma cells, and other cellular elements are to be found

6 Marinesco (1918 *a*) recorded the presence of corpuscles staining by Gram and Leishman about 1 μ in diameter, either isolated or in pairs, in the perivascular

Blood The reports of blood examination seem all to refer to cases of later date, but they are included here for convenience As a general rule the white blood count has been found normal, but on occasions a neutrophil leukocytosis up to 20,000 per c.mm. has been recorded, and the more acute the case the greater this leukocytosis (Berger and Untersteiner, 1925). It has been said that the presence of eosinophilia, even up to 50 per cent, is a sign of good prognostic significance (Bohrod *et al*, 1931).

Duration of Illness

The average length of illness in patients who recovered was as follows (Parsons, MacNalty, and Perdrau): age up to 10, 4 to 7 weeks; age 10 to 15, 11 to 12 weeks, age 15 to 30, 5 to 8 weeks, over 30, 8 to 10 weeks.

In cases with a fatal result the same authors stated that 50 per cent. died within 2 weeks and 80 per cent within 4 weeks.

Sequelae

These were recorded by a number of authors, particularly MacNalty (1918) and Parsons, MacNalty, and Perdrau, the following being commonly observed failure of the general health, disorders of the mind, salivation; tremors, monotonous speech, ocular defects, pareses of face and limbs, athetosis, parkinsonism, with facial mask, neck bent forward, hypertonic and tremulous arms, and festinant gait In the Sheffield outbreak of 1924 there was a definitely increased liability to parkinsonism in males (Hall and Yates, 1926) Parkinsonian cases were probably infectious, although cases of contact infection were only rarely recorded (e g, by Crouzon and Horowitz, 1929, Netter, 1933).

Mortality

The case mortality rate for 1,273 cases notified during 1919 and 1920 was 48 3 per cent (Parsons, MacNalty, and Perdrau, 1922). There was no appreciable difference in regard to the sexes. With regard to age, in infants (0–1 year) the percentage dying was high (75 per cent), but this percentage decreased to 33 per cent for the age group 10 to 15 years Thereafter the percentage rose steadily to attain 80 per cent in the age group 70–80 The authors quoted regarded the high case mortality rate as fallacious, owing to the number of mild and abortive cases either missed or not certified as encephalitis lethargica. They gave the case mortality rates from numerous other countries, of which may be mentioned the United States (1918–19) 34 per cent, France (1919–21) 37 per cent, and by contrast, South Africa (native patients, 1920) 92 per cent.

CLINICAL FEATURES OF THE LATER CASES (1920 TO 1924)

In 1920 the disease altered in its characteristics in various directions, thus (*a*) pyrexia was slight, (*b*) ocular manifestations were slight, (*c*) lethargy gave place to excitement (Bramwell, 1921)

In 1923 a representative outbreak in Liverpool was studied by Stallybrass Of 130 cases 45 per cent were oculolethargic, 14 per cent myoclonic, 25 per cent. choreiform, and 16 per cent psychomotor Evidences of motor excitement were thus somewhat commoner than lethargy and ocular palsies, and sleeplessness, hypermotility, twitchings, grimacings, tachypnea, and occupational delirium were all found. Parkinsonism was a common sequel The case mortality rate was 40 per cent

A similar outbreak in Sheffield in 1924 was recorded by Hall and Yates (1926)

CLINICAL FEATURES AT THE PRESENT DAY

At the present day sporadic cases of encephalitis lethargica are seen from time to time. The commonest type is comparatively mild, and ambulant cases are met with Sometimes only the onset of parkinsonism shows the true nature of some

mononuclears Hemorrhages occur in the pia arachnoid over the hemispheres, brain stem, and cord, and there are usually collections of mononuclear phagocytic cells around the hemorrhage. Vascular siderosis is usually present in the anterior half of the globus pallidus.

Changes are widespread and the substantia nigra, nuclei of the brain stem, and the basal ganglia all show varying degrees of involvement. The following areas are involved in almost every case. red nucleus, globus pallidus, substantia nigra, dentate nucleus, and olives. The primary change is a cell destruction which is followed by growth of the astrocytes. This cell destruction is progressive and indicates the continued activity of the causal agent. The destruction is patchy and different cell masses are affected more or less independently, thus, one nucleus may be severely involved while a neighbor is unaffected The process differs from that observed in the acute stage, in that cell destruction and reparative gliosis are features, rather than activity of the cellular elements derived from the blood and vessels The change is a simple one and specific only as regards the site of involvement.

C. Pathology of the Remainder of the Body

Boyd (1920 *a*, *b*) described vascular and degenerative changes in the kidneys, and, rarely, widespread hemorrhages in the serous membranes. Netter (see Netter, Cesari, and Durand, 1921 *a*, *b*) attached great importance to round cell infiltrations of the salivary and parotid glands Apart from these lesions the remainder of the organs is usually found to be healthy.

EPIDEMIOLOGY

The information contained in this section has been summarized from the extensive work of the following investigators, mainly during the years 1918 to 1926 when the disease assumed epidemic proportions· James (1918 *a*, *b*), MacNalty (1919, 1919–20, see also 1937), Bernard and Renault (1920), Netter (1920 *c*, see also 1933), Kling and Liljenquist (1921); Smith (1921), Parsons, MacNalty, and Perdrau (1922), Wynne (1926) The statistical aspect of the disease has been dealt with by Stocks (1932).

Topographical Distribution

The distribution of cases followed closely that of the population, and therefore the majority of cases was urban rather than rural. No part of the country was particularly affected, and in individual towns there was no significant grouping of cases in tenements, streets, or particular localities

Possible Association with Influenza

On account of the pandemic of influenza more or less coexistent with that of encephalitis lethargica, it was only natural that some association between the two conditions should be sought Two main views were advanced, first, that influenza rendered the individual susceptible to encephalitis, and secondly, that encephalitis was an influenzal manifestation Without entering into the subject in detail (see Parsons, MacNalty, and Perdrau, 1922, for a longer discussion of the subject, also Jordan, 1927) it is sufficient to say that the balance of opinion was in favor of the unlikelihood of both views Influenza did not predispose to encephalitis and encephalitis was certainly not a nervous form of influenza More recently, however, it has been reported that influenza virus may occasionally cause an encephalitic illness (Brown *et al*, 1945).

Age, Sex, and Social Conditions

While the disease occurred from infancy to advanced old age, it was found chiefly in those approaching adult life (i e., up to 20 to 30), and thereafter there was a diminished liability to infection.

region. He suggested that these structures were protozoa. Levaditi and Harvier (1920 *e*) described corpuscles 4 to 5 μ in diameter in perivascular spaces, composed of a central chromatin mass and a blue cytoplasm.

7. Boyd (1920 *b*) pointed out that in a number of cases where there was cranial nerve disturbance, the corresponding nerve fibers were found to be pressed upon by dilated vessels, the nuclei being healthy.

8 Focal edema in the brain stem and basal ganglia may occur.

9. Proliferation of the lining cells of the aqueduct of Sylvius has been described The cavity becomes partly occluded by filiform digitations observable symmetrically at the posterior angles (Ravairt, Nayrac, and Dubois, 1929).

10. The pia mater shows considerable vascular dilatation, and hemorrhages are also found.

11. Attempts have been made to account for the lethargy on the basis of specific pathological lesions, but no satisfactory results have been achieved. It is probably not dependent on increased intracranial pressure (Bramwell and Miller, 1920), and may be due to interruption of the afferent stimuli passing to the thalamus.

12. The most important inclusion bodies are those known as the "minute bodies" of Da Fano (Da Fano and Ingleby, 1919, Da Fano, 1921 *a*, *b*, *c*, Shaw, 1921; Hume, Nattrass, and Shaw, 1922; and many others) The bodies occur in the cytoplasm of the nerve cells, stain black by Bielchowsky's silver stain, and are best demonstrated by toluidine blue or polychrome methylene blue. In the unstained state they appear yellowish-brown, do not give the test for masked iron or sulfur, are insoluble in acids and alkalies, and do not stain with Sudan or Scharlach Usually each body (or pair of bodies) is surrounded by a halo and measures 0.4 μ in diameter. The cells containing these bodies rapidly degenerate Bodies may also be found extracellularly Similar bodies have been found in the salivary glands. Da Fano bodies of precisely similar type occur in experimental herpetic encephalitis of animals. The bodies have not been sufficiently studied for a definite opinion as to their nature to be expressed, but they may well represent aggregates of the causal virus agent.

Dawson (1933, 1934) described eosinophilic intranuclear bodies which were found either with or without intracytoplasmic bodies The intranuclear bodies were round or oval and varied from twice the size of the nucleolus almost to that of the nucleus itself The intracytoplasmic bodies occurred as small pink masses or larger irregular structures The bodies were found in the motor cortex, red and caudate nuclei The intranuclear bodies would appear to be of the Lipschutz type and similar to those found in herpes

The majority of observers holds the histological picture to be specific, and yet at the same time to be somewhat similar to that of encephalitis due to African trypanosomiasis On the strength of this similarity Bassoe and Hassin (1919) suggested that a protozoon was the cause of encephalitis lethargica, but this opinion has never been seriously entertained

B. Chronic Infections

Microscopically, as the disease progresses to chronicity, alterations in the nerve cells are accentuated, while the perivascular reaction largely subsides The histological picture has been described by various authors (see, e g, Levaditi and Harvier, 1920 *e*, Reynolds and Slater, 1929–30, Carmichael, 1930–1). Neustaedter and Liber [illegible] of their own The following
how conspicu-
nd dendrites),
re is extensive
destruction of the large multipolar cells in the substantia nigra A purely reparative *proliferation* of the astrocytes occurs as a late part of the picture. The blood vessels, especially in the substantia nigra, show well marked perivascular infiltration with

have both been advocated. In all probability none of these measures exerts more than a temporary and slight effect on the course of the disease. Successful results have been reported, (a) after injections of the sera of rabbits hyperimmunized against herpes, (b) after immunization against a neurotropic herpes strain, and (c) after injection of a *formolized* virus vaccine (Neal and Bentley, 1932, Neal, 1934).

THE ETIOLOGY OF ENCEPHALITIS LETHARGICA

Encephalitis lethargica presents many of the characteristic features of an infective disease, thus, the pathological picture is typically infective, and cases of contact infection have been recorded on a number of occasions. The causal agent is, however, as yet unidentified.

The main theories that have been advanced are that encephalitis is caused by (1) a toxicosis, (2) a cultivable bacterium, (3) a virus other than that of herpes, (4) the virus of herpes.

1. The Rôle of Toxicosis

Veillard (1929) suggested that the disease is essentially a toxemic condition from the alimentary tract. Beans, corn, and other substances were alleged to be acted on by bacteria and in this way an encephalitogenic agent was produced.

A number of authors suggested that the encephalitogenic "toxin" might be produced from organisms growing elsewhere in the body, i.e., in the nasopharynx (Martin, 1918) or the gallbladder (Salzman, 1924, 1925).

Finally, various alleged deficiences in the liver, or sugar or nitrogen metabolism have been blamed (e.g., by Meyer-Bisch and Stern, 1923, Buscamo, 1924, Runge and Hagemann, 1924–5).

These theories are, in our opinion, totally inadequate to explain the various features of encephalitis lethargica, particularly its frequent occurrence in epidemics.

2. Cultivable Micro-organisms as the Causal Agents

von Wiesner (1917) injected a monkey subdurally with brain emulsion from a fatal case of von Economo's. The animal died 46 hours after the injection, having been somnolent and partially paralyzed. A gram-positive diplococcus was isolated.

Strauss, Hirshfeld, and Loewe (1919) isolated a filtrable agent from cases of encephalitis which produced lesions in a monkey resembling epidemic encephalitis. Loewe, Hirshfeld, and Strauss (1919) carried out further experiments by injecting suspensions of nasopharyngeal mucosa into rabbits and monkeys. Some of the latter developed lethargy, fever, and ptosis, while histologically the rabbits' brains showed lesions similar to those of man.

Loewe and Strauss (1919, 1920) obtained growths of small globoid bodies in Noguchi's spirochete *medium* from the brains of inoculated animals and from human cerebrospinal fluid. Numerous other workers isolated organisms cultivable in ascitic fluid or other rich media from cases of encephalitis, usually from the nasopharyngeal washings, and less often from the cerebrospinal fluid, blood, or brain (e.g., Bastai, 1920, 1921 *a*, *b*, Ottolenghi, d'Antona, and Tonietti, 1920, Maggiore and Sindoni, 1921, Thalhimer, 1921, 1922, Rosenow, 1924, Evans and Freeman, 1926, and numerous others described in the Matheson Survey, 1929). McCartney (1924) was not, however, able to isolate any globoid bodies from cases of encephalitis, and suggested that the various organisms described were accidental contaminants.

There have been many fallacies in the above reports, which are not generally accepted. Many were undoubtedly working with contaminants which either gained access during the postmortem in the case of brain, or else were present in the naso-

The general consensus of opinion indicated that the sexes were equally affected Social conditions such as *overcrowding, insanitary environment, social status,* and occupation played no part in determining the incidence of the infection

The Method of Spread

Routine epidemiological investigations of the possible rôle of food, milk, water, insect vectors, and fomites yielded completely negative results All observers agreed on the rarity of case-to-case infection, and the epidemic was essentially composed of a large number of sporadic cases. Thus, Smith (1921) detected no case of infection amongst 900 contacts in the United States, and in France, Bernard and Renault (1920) found no evidence of direct contagion resulting from any one of 400 cases

A number of well-authenticated cases of contact infection has, however, been recorded, thus, MacNalty (1919–20) investigated an outbreak in a girls' home in Derby (England) where 12 cases occurred among 22 inmates. He suggested that the infection had arisen from a carrier or mild abortive case. Zinsser (1928) also recorded an institutional outbreak. Cases of infection in families and other contacts have also been recorded on occasions (MacNalty, 1919, Netter, 1920 *c*, see also 1933, Kling and Liljenquist, 1921, Stallybrass, 1923, Wynne, 1926, Crouzon and Horowitz, 1929).

Netter stressed the part played by the saliva in the spread of contagion, but this was not generally accepted as an adequate explanation.

It is difficult to see how the virus spread from case to case unless it were by the air Indeed, were it not for the well-authenticated cases of contact infection the very infectivity of encephalitis lethargica might have been questioned on purely epidemiological grounds. Suspicion was naturally directed toward the part played in the spread of infection by carriers, mild, abortive, or parkinsonian cases, but even these would not account for the majority of instances of the disease.

If the method whereby the virus spread from case to case was obscure, still more so was the route whereby it actually reached the central nervous system. *Probably the portal of entry was the respiratory tract, but whether the virus* reached the brain via the blood stream or via the olfactory nerves and their perineural spaces was not known An interesting observation tending to support the party played by the nasal mucosa was made by Bramwell (1921). A patient had suffered from a nasal discharge due to a polypus for some considerable time Eventually the obstructing tumor was removed, presumably severely traumatizing the nasal mucosa, and a few days later a typical encephalitis lethargica developed

Numerous cases of transmission of the disease by an encephalitic mother to her newborn baby were recorded (Harris, 1918, MacNalty, 1919, Sousa, 1920, Parsons, MacNalty, and Perdrau, 1922) Usually the child developed somnolence, myoclonus, retention of urine, or other symptoms shortly after birth, but eventually recovered

Prevention

As the disease is manifestly of such low infectivity it is questionable whether elaborate precautions need be taken to isolate the patient, or *quarantine contacts*, but in actual practice the patient is usually isolated in hospital and children kept from school for some weeks (see *Memorandum*, 1927)

TREATMENT

Convalescent serum has been used by certain workers, but evidences of beneficial effect would appear to be only slight (Grunewald, 1920, Brock, 1931, Lapage, 1933) Convalescent cerebrospinal fluid has been injected subcutaneously (Collitino and Vegni, 1920) Intraspinal injection of the patient's own serum (Brill, 1920), and subcutaneous injection of the patient's own blood (Bourges and Marcandier, 1920)

any less valid on this account, and suggested that the control animal had become spontaneously infected with the virus of encephalitis lethargica. McIntosh's results have not received general confirmation and meanwhile must be accepted with some reserve

Later, McIntosh (1923) suggested that rabbits might be used for diagnosis. He injected saline suspensions of basal ganglia and cerebrospinal fluid intracerebrally. Positive results consisted in the development of symptoms 48 hours later with death in 5 to 6 days. Histologically, the lesions were said to be typical of encephalitis. Such results were achieved with 3 out of 5 brain emulsions, and 1 out of 4 cerebrospinal fluids tested A further paper was published in 1927, and with Scarff in 1928

These results are not easy to interpret, and are contrary to the great bulk of experimental work detailed elsewhere in this chapter, in which a virus pathogenic for the rabbit was isolated only with extreme rarity, or by a special technique

(c) *Miscellaneous results.* A large number of workers (see Matheson Surveys, 1929, 1932) has reported the production in animals of keratitis, encephalitis, or other lesions with material from cases of encephalitis. This material usually proved sterile on ordinary media and had often been filtered The experiments were usually very inconclusive, however, and no well-recognized strain of virus emerged The effects noted were probably in the main nonspecific, or else due to an unrecognized bacterial contaminant or to spontaneous encephalitis Probably in some cases a weakly virulent strain of herpes virus was actually present It has even been suggested that the mumps virus is the cause of encephalitis lethargica, but this has not received any serious consideration (Gundersen, 1927)

4. The Herpes Febrilis Virus

There is no doubt that herpes is capable of causing a fatal encephalitis in man, inclusions can be found readily in the nuclei of the brain cells, and virus can be isolated (see Ch. XVII). In general, however, herpetic encephalitis, as at present observed, appears not to resemble encephalitis lethargica very closely.

(A) ISOLATION OF HERPES VIRUS FROM CASES OF ENCEPHALITIS LETHARGICA

A number of strains of virus has been isolated from cases of encephalitis lethargica since the first report by Levaditi and Harvier (1920 *a*). In almost all these cases the virus has been carefully studied by cross immunity and other experiments, and its identity with herpes febrilis rendered almost certain. (The properties of these strains are described below in section C) It is not clear whether all the strains to be mentioned were isolated from typical cases of lethargic encephalitis Some of the cases may have been more acute, resembling herpetic encephalitis

1 *Levaditi's strains* Levaditi isolated 3 distinct strains of virus and these will be considered in some detail, as much work has been carried out, particularly on one of these strains (C)

(*a*) *Strain* C was isolated from the brain of a case of encephalitis by rabbit injection (Levaditi and Harvier, 1920 *a* and *e*) Unfortunately, however, the patient suffered at the same time from facial herpes (Rouillard, 1921), and as it is well known that the virus may be found in the cerebrospinal fluid in herpetic eruptions (see Ch XVII), it is difficult to attach much importance to the fact of this isolation However, the great bulk of the French work has been carried out on this strain, and on the basis of its undoubted identity with the virus of herpes, Levaditi and his associates have claimed that encephalitis is a herpetic infection. Strain C has been maintained by cerebral passage in rabbits since its original isolation, and definite loss of virulence has occurred Originally strongly kerato- and encephalito-genic, the strain now fails to produce keratitis, and only very rarely causes encephalitis This loss of virulence has been studied by a number of authors and the weak strain used for various experiments, more particularly in connection with "auto-

pharynx quite apart from encephalitis. Certain workers were also unaware of the existence of spontaneous encephalitis of rabbits.

3. The Rôle of Viruses other than Herpes

(*a*) *Work of Kling, Davide, and Liljenquist* (1922 *a–f*). These Swedish workers reported the isolation of a strain of virus from cases of encephalitis lethargica which showed no cross immunity reactions with herpes febrilis. It did not produce keratitis on corneal inoculation, but the rabbits died some weeks later from encephalitis It was heat resistant. Although interest was aroused at the time, this soon lapsed when Levaditi, Nicolau, and Schoen (1924) showed that the "virus" was only that of spontaneous encephalitis (*E. cuniculi*), from which infection Kling's rabbits were evidently suffering. However, as Doerr and Zdansky (see below) were able to isolate herpes virus from material sent by Kling, it is probable that he may actually have been

(*b*) *Work* … McIntosh (1918), McIntosh and … om the Derby institution outbre … ycerol for 14 days was used for inoculation, but it was said to be very soft and decomposed One rhesus monkey was injected with unfiltered brain material intracranially and intraperitoneally. After about 14 days it became drowsy and weak, and died 7 days later Postmortem, tuberculosis of the lungs was found, and examination of the brain showed only a few small hemorrhages in the midbrain. A *Cercopithecus patas* monkey was similarly injected with a Berkefeld filtrate. After 7 days it developed an excited fit but recovered the next day. Six weeks after the injection, it had a severe fit and remained rigid and tremulous and disinclined for food until death almost 2 months after injection.

Microscopically, the central nervous system showed active inflammatory change in the posterior half of the left basal ganglia and junction of left basal ganglia and midbrain. Slight perivascular infiltration in the cerebral pia was also noted The right globus pallidus showed engorgement, hyaline thrombosis, and perivascular hemorrhages. There was evidence of *ante mortem* neuronal degeneration in the anterior horn of the lumbar cord, cerebrum, and elsewhere.

McIntosh (1920) continued his previous experiments by injecting 2 cynomolgus monkeys intracerebrally and intraperitoneally with the brain of the previous patas monkey One cynomolgus monkey remained quite healthy The other remained well for about 3½ months, when muscular tremors were noted Ten days later the animal became somnolent, and in another 2 weeks the hind legs were weak. One month after the first appearance of symptoms it became very excited and had an epileptiform fit. Finally, 5 months after injection it became so weak that it was killed

Microscopically, acute inflammatory changes were observed in the brain, mainly in the basal ganglia Glial proliferation, perivascular infiltrations with lymphocytes, congestion, perivascular hemorrhages, and degenerative changes in the ganglion cells were all noted

The brain was passaged to a baboon, but it died after 4½ months without microscopic evidence of encephalitis

Rabbits were injected intracerebrally with monkey brain, one developed encephalitic symptoms and was killed in 8 days Histologically, its brain showed some lymphocytic infiltration in the meninges and perivascular sheaths

A cynomolgus monkey used as a control developed symptoms precisely similar to the one injected Histologically, its brain showed lesions in the basal ganglia typical of encephalitis lethargica.

According to the usual manner of interpreting animal experiments, the occurrence of this spontaneous encephalitis in the control would have seriously lowered the value of McIntosh's results. He did not, however, think that his results were

sterilizable neuro-infections (Levaditi); (2) the effect of glycerol in "unmasking" the virus, and the action of "aggressins" (Perdrau).

1. The theory of autosterilizable neuro-infections.

This theory has been largely propounded by Levaditi and his colleagues L dit and Nicolau (1924) showed that cynomolgus monkeys injected with v isolated from encephalitis contracted an encephalomyelitis, histologically typ yet apparently the brain was avirulent for rabbits This observation led Leva Sanchis-Bayarri, and Schoen (1928) to introduce the term "autosterilizable ne infections" to cover those cases where virus could not be isolated, althoug presence was anticipated These workers suggested that the virus primarily tacked and destroyed nerve cells, and that the body then reacted to defend it This reaction, shown by cellular infiltrations, neuronophagia, and gliosis, m result in the death of the virus But in brain where so much of the tissue is to life this reaction produced a fatal result, with consequent failure to isolate living virus. It would appear that most of these negative results were found in case of animals which had died after 3 or more weeks' illness, in which the v had been killed by the cellular reaction, the extent of this reaction making the tinuance of life impossible Further, the strain of virus used to inoculate such mals has usually been of low virulence (strain C) Similar conclusions have arrived at by later workers (Lépine, 1928, Levaditi, 1929, Lépine and Schoen, 19

It is questionable, however, whether the absence of the virus from such b is not merely apparent, for Lépine (1930), by cataphoresis, has separated off a virus from brains apparently inactive on intracerebral injection Also, it is prob that, although a brain may appear avirulent at first, after serial passage it yield virus.

At any rate, as many of the human cases examined have been acute in nat it is unlikely that this mechanism could wholly account for the total failure o many workers to isolate the virus

2. Effect of glycerol.

It has been shown that virus isolated from brains of cases of encephalitis is n active after treatment in glycerol than before (Levaditi, 1922, Berger, 1923, drau, 1925 *b*). Similar observations have been made with herpes virus isolated f herpetic vesicles (see Ch XVII)

It appears as if some substance exists in fresh brain which exerts an inhibi effect on the virus, and that glycerol acts by destroying this inhibitory factor many investigators have used fresh human brain or cerebrospinal fluid for t animal inoculations, this fact may undoubtedly explain some of the failures to late a virus.

Perdrau (1925 *b*) attempted isolation from human brains by stimulating virus therein with "aggressins." This method developed directly out of work herpes virus (1925 *a*, see Ch XVII) in which he found that the brains of her immune rabbits had an "aggressin-like" action when added to virus, on intracere injection of the mixture, the usual incubation period was markedly shorte Perdrau's method consisted in placing the midbrain and basal ganglia in glyc for a week or so and then stimulating the virus supposed to be therein by on three procedures (*a*) By adding the brain of a herpes-immune rabbit (the b having been in glycerol for 2 to 3 weeks) to the human brain and injecting mixture The herpes-immune brain was said to contain aggressins, and by this m Perdrau succeeded in isolating herpes virus from the brain of a case of encephal which proved avirulent in the fresh state (*b*) By producing the aggressin in animal to be injected The human brain was injected intradermally and the foll ing day a further injection (with herpes-immune brain added) given intrac brally, on each of the next 7 days a cutaneous injection was given by deep scarif

sterilizable neuroinfections" (Levaditi, Sanchis-Bayarri, and Reinié, 1927; Levaditi, Sanchis-Bayarri, and Schoen, 1928, Levaditi, Lépine, and Schoen, 1929, Lépine and Schoen, 1930). Olitsky and Long (1928) also studied the virus and succeeded in infecting guinea-pigs.

(*b*) *Strain CH* (Levaditi, Harvier, and Nicolau, 1922). This strain was isolated from the nasopharynx of a case of encephalitis The strain produced keratitis on corneal inoculation, sometimes with the development of encephalitis, and proved to be of the same biological nature as strain C. Again, however, the nasopharynx is known to contain herpes virus in normal persons and so this strain also is suspect.

(*c*) Levaditi and Harvier (1920 *c*) also isolated a third strain from the brain of a choreiform case of encephalitis by intracerebral injection of rabbits This strain was of low virulence, however, and was not maintained, which is unfortunate, as *its claim to consideration would seem to be more definite than strains C or CH*

2. *Netter, Cesari, and Durand* (1921 *a*) isolated a weakly virulent herpes virus from the brain of cases of encephalitis on two occasions.

3. *Basel strains.* By the work of Doerr and his associates, 3 strains were isolated in Basel. Strain 1 was isolated from the cerebrospinal fluid of a case, by intracranial injection of a rabbit, and was extensively studied (Doerr and Schnabel, 1921 *a, b, c*). Strain 2 was isolated from the brain by intracerebral injection of rabbits (Doerr and Berger, 1922). Strain 3 was isolated from the brain (after 14 days in glycerol) by Berger (1923).

4. *The Berlin strain* was isolated by Schnabel (1922) from the cerebrospinal fluid, by intracranial injection of rabbits

5. *The Vienna strain* was isolated from the cerebrospinal fluid, by intravenous injection of rabbits (Lauda, 1923, Luger and Lauda, 1924).

6. *Doerr and Zdansky's (1924) strain* was isolated from brain sent (in glycerol) by Kling, using corneal inoculation of rabbits.

7. *Bessemans and van Boeckel's (1923 a, b) strain.* These workers injected a number of rabbits with brain, cerebrospinal fluid, and nasopharyngeal secretion from cases. From 7 rabbits herpes virus was recovered, but it is uncertain how many cases this represented. At any rate, from a few cases these authors were able to isolate herpes virus

8. *Danila and Stroe* (1923 *a, b*) isolated an encephalitogenic, nonkeratogenic virus from the nasopharynx of a case, but as there was a coexistent herpetic eruption, this result should be interpreted with considerable reserve.

9 *Sicard, Paraf, and Laplane* (1923) isolated a strain (by intracerebral injection of rabbits) from the mesencephalon of a case of parkinsonism

10. *Perdrau's (1925 b) strains.* By means of a technique specially designed to recover herpes virus from the brain (see below) Perdrau recovered 3 strains, one of which (E L. 1) was more extensively studied than the others

11. *Gay and Holden* (1932–3, 1933) isolated herpes virus from the basal ganglia of a case of parkinsonism by injection of rabbits and monkeys They obtained a similar virus from a case of ascending myelitis following a monkey bite, but this case should not be accepted as one of encephalitis lethargica, although Gay and Holden appear to have regarded it as such

12. *Kreis* (1938) isolated herpes virus from the saliva of 3 cases of postencephalitic parkinsonism

(B) EXPLANATIONS TO ACCOUNT FOR FAILURES TO ISOLATE HERPES

Numerous workers (presumably many never published their results) have entirely failed to isolate a virus from cases of encephalitis lethargica (e g, Ford and Amoss, 1924, Dible, 1925, Flexner and Amoss, 1925, Douglas, 1926, Crouzon and Horowitz, 1929, Dawson, 1933, Rupilius and Szekely, 1934) To explain these results, those who have been more fortunate have advanced hypotheses (1) auto-

vier, and Nicolau, 1921 *a, d*, 1922). The virus passed through the placenta and was isolated from the brain of a fetus by Levaditi, Harvier, and Nicolau (1921 *c*).

2. *Guinea-pigs* were susceptible to intracranial injection of rabbit passage virus (Levaditi and Harvier, 1920 *b*, Doerr and Schnabel, 1921 *a, b, c*). Working later, Olitsky and Long (1928) found that strain C could not be transmitted by direct intracerebral injection. They succeeded in transmitting the infection by the following technique The animals were inoculated intracerebrally, and the left cornea was scarified with virus, and on the 3rd day the right cornea also was scarified On the 6th to 7th day the animal developed the typical syndrome of circling, tremors, contractions, urinary retention, and marked salivation The virus was transmitted by passage, and after 3 passages it was merely necessary to inject the material intracerebrally, the additional scarification becoming unnecessary.

3. *Mice* were found to be susceptible to rabbit passage virus on intracerebral, intraperitoneal, and subcutaneous injection (Levaditi and Harvier, 1921 *a*, Doerr and Schnabel, 1921 *a, b, c*, Levaditi, Harvier, and Nicolau, 1922).

4. *Monkeys*. It was found (Levaditi and Nicolau, 1924) that intracranial injection of certain monkeys and chimpanzees caused the development of an encephalitis, acute or chronic, and histologically typical.

Morphology and filtrability of the virus.

Inclusion bodies. The *neurocorpuscules encephalitiques* (Levaditi, Harvier, and Nicolau, 1921 *a*, 1922) occurred in the nuclei (rarely in the cytoplasm) of nerve cells in the hippocampus of infected rabbits. They measured 1 μ in diameter and were surrounded by a clear halo. Schnabel (1924) reported that typical Lipschütz bodies developed in the epithelial cells of the infected rabbit cornea

The virus passed through a Chamberland L_1 and L_3 (Levaditi and Harvier, 1920 *a, e*, Levaditi, Harvier, and Nicolau, 1921 *b*).

Culture.

It was shown that the virus conserved its virulence at 37° C in tissue culture as long as the cells were still viable (Levaditi and Harvier, 1920 *e*), but more extensive experiments on actual propagation in tissue culture do not appear to have been carried out

Reaction to physical and chemical agents.

The virus was destroyed at 55° C. in half an hour

After desiccation *in vacuo* the virus conserved its virulence for at least 44 days, but if desiccated over sulfuric acid, for not much longer than 4 days (Levaditi, Harvier, and Nicolau, 1922)

Various chemical substances given to rabbits increased the virulence of virus subsequently injected, such as chloral, chloroform, and ether (Levaditi, Harvier, and Nicolau, 1922), alcohol and thyroxin (Levaditi, Lépine, and Schoen, 1930) Raising the animal's temperature achieved the same result (Levaditi, Lépine, and Schoen, 1929)

The virus was, of course, glycerol-resistant and this effect has already been discussed in detail The virus did not readily diffuse out of a piece of brain placed in glycerol (Levaditi, Harvier, and Nicolau, 1921 *a*)

The virus was adsorbed by animal charcoal and kaolin (Levaditi and Nicolau, 1923 *a*) and was susceptible to bile (Levaditi and Harvier, 1921 *b*, Levaditi, Harvier, and Nicolau, 1922)

The virus survived in the tissues of infected animal cadavers for at least 48 hours (Levaditi and Harvier, 1920 *e*)

The virus conserved its activity at room temperature for at least 50 days in water and 86 in milk (Levaditi, Harvier, and Nicolau, 1922, Levaditi and Harvier, 1921 *a*)

tion (the scrapings of each previous scarification being used for the next in series) By this means Perdrau isolated herpes from another case (*c*) Finally virus was isolated in a third case by repeated intradermal passage of human brain material in rabbits.

(C) PROPERTIES OF HERPES VIRUS ISOLATED FROM CASES OF ENCEPHALITIS LETHARGICA

Human experiments.

Schnabel (1923) inoculated himself on the skin with rabbit brain material (Berlin strain) with the production of only a slight reaction. Bastai and Busacca (1924, 1925) injected the same virus into the subarachnoid space of one patient with negative results, and into the skin of three others with only a slight reaction

Strain C was injected intrathecally (Nicolau and Poincloux, 1924–5), but could not be recovered by animal experiment even 8 hours later. It is evident, therefore, that these experiments do not strengthen the position of herpes virus as the cause of encephalitis lethargica.

Animal experiments.

1. *Rabbits.* (*a*) *Corneal injection* (Levaditi and Harvier, 1921 *a*, Levaditi, Harvier, and Nicolau, 1921 *a*, 1922, Doerr and Schnabel, 1921 *a*, *b*, *c*; and many others). Keratoconjunctivitis was found to develop 24 to 48 hours after corneal scarification, and, in the case of a virulent strain, spread occurred to the brain (within 48 hours) via the optic and trigeminal nerves. The animal died with the typical encephalitic syndrome in 6 to 8 days after inoculation.

(*b*) *Intracranial injection* (Levaditi and Harvier, 1920 *a*, *e*, Doerr and Schnabel, 1921 *a*, Levaditi, Harvier, and Nicolau, 1921 *a*, 1922, Schnabel, 1922, Luger and Lauda, 1924, Perdrau, 1925 *a*, *b*). Following injection of human material there was usually an incubation period of some 8 days before symptoms developed, but in the case of rabbit passage virus this was shortened to 4 to 6 days. The symptoms were typical of herpetic encephalitis, tremors, fits, and somnolence all occurring

Histologically, brains of animals dead of encephalitis showed lesions which were widespread, though most marked in the hippocampus. There was much destruction of nerve cells with tigrolysis, neuronophagia, and infiltration with polymorphs Miliary abscesses might even be found, and the acuter the inflammation the greater the number of polymorphs present In cases where death had been more delayed the main cellular infiltration was mononuclear. The meninges showed infiltration with lymphocytes, macrophages, and plasma cells, hemorrhages were also found Perivascular cuffing with mononuclears and polymorphs was also observed in the earlier stages.

(*c*) *Cutaneous injection* (Levaditi, Harvier, and Nicolau, 1921 *f*, 1922). A papulosquamous eruption followed cutaneous injection, and the animal died from encephalitis Paresis of the hind limbs might be observed

(*d*) *Other routes of injection.* The rabbit could be infected by the following routes nasal (after trauma or instillation of croton oil), anterior chamber of eye, peripheral nerves, and testicle (Levaditi and Harvier, 1920 *a*–*e*), intratracheal, intramuscular, intraperitoneal (Levaditi, Harvier, and Nicolau, 1921 *f*, 1922), intravenous (Zdansky, 1923, Luger and Lauda, 1924) The following routes gave negative results intragastric, via the salivary gland, and subcutaneous (Levaditi and Harvier, 1920 *e*).

Distribution of virus in the infected rabbit. Examination of an animal dead from infection showed that the virus was present in the brain and spinal cord, but not in the cerebrospinal fluid, blood (yet the polynuclear count might reach 18,000 per c mm.), marrow, internal organs, testicle, salivary glands, spinal ganglia, lymph glands, or nasal mucosa (Levaditi and Harvier, 1920 *c*, *d*, *e*, 1921 *b*, Levaditi, Har-

on *a priori* grounds no virus obtained from the nasopharynx of encephalitic patients can be seriously considered as the etiological agent. The great majority of encephalitis strains of virus has been isolated from the brain, but even here it is possible that the virus might be an accidental invader, and not the etiological agent. Again, the cases described as lethargic encephalitis may have been herpetic encephalitis.

2. *Rarity with which herpes virus has been isolated from encephalitis lethargica.* Only on about 20 occasions has herpes virus been isolated from cases of presumed encephalitis lethargica. The theories brought forward in explanation have already been discussed. The theory of "autosterilizable neuro-infections" rests on no very strong basis of experimental evidence. At any rate, it would not explain the absence of virus from the brains of chronic encephalitics, where the whole pathological process indicates the continued activity of the virus. The demonstration, by Perdrau and others, that glycerol treatment may be needed to unmask a latent virus may certainly explain a number of failures to isolate virus from fresh brain. Yet if herpes virus is the cause of encephalitis lethargica, then it is almost unique in the difficulty with which it can be isolated. In the case of rabies, poliomyelitis, and St. Louis encephalitis, no difficulty is experienced in isolating by animal injection the characteristic causal agent. Moreover, in herpetic eruptions the virus is readily obtainable and no theories have had to be adduced to explain failures. The results of attempts to isolate herpes virus by animal experiments must, therefore, be regarded as very unsatisfactory.

3. *Clinical observations.* Although herpes virus can cause a severe involvement of the central nervous system (see Ch. XVII), such cases do not resemble encephalitis, and this is hardly to be expected if the same agent is responsible for the two conditions.

4. *Pathological observations.* Undoubtedly pathological observations do much to support herpes virus as the etiological agent of encephalitis. There is considerable similarity between the pathological picture of acute encephalitis in the human being (see above) and that observed in the rabbit injected intracranially with herpes virus. The main tissue changes are of the same order, and the "minute bodies" or inclusions of Da Fano are found in both (see Da Fano, 1923). Intranuclear inclusions occur in herpetic encephalitis and have been described in lethargic encephalitis. There is, however, even more histological similarity between human brains and those of partially immunized rabbits suffering from herpetic encephalitis (Perdrau, 1925 *a*). Herpetic infection of monkeys also resembles human encephalitis very markedly both clinically and histologically.

It is evident, therefore, that herpes is able to produce experimentally in animals lesions of a type almost identical with those of human encephalitis lethargica.

5. *Suggested methods of transmission of the virus of epidemic encephalitis.* It has been shown that herpes strains isolated from cases of encephalitis can survive for weeks in milk or water. Epidemiological investigations have definitely shown, however, that the disease is not so spread.

On the basis of animal experiment it has been suggested that the herpes virus may be spread by sexual intercourse. A local lesion results and encephalitis follows (Levaditi and Nicolau, 1923 *b*).

Simmons, Kelser, and Cornell (1933, 1934) suggested that the virus might be spread case to case by means of *Aedes aegypti*, but again epidemiological investigations have failed to incriminate any insect vector.

Levaditi and Netter both stress the importance of the saliva in the spread of infection. While Netter believes that infection may result from contact with an infected person through the medium of the saliva, Levaditi stresses more the rôle of endogenous factors.

Levaditi has stated that 80 per cent. of persons carry a keratogenic salivary virus and 15 per cent. an encephalitogenic virus, both being typical strains of herpes

Immunity.

The most important work was directed toward proving the identity by cross immunity experiments of virus strains isolated respectively from encephalitis and known herpetic vesicles. A typical experiment which proved this identity was that of Doerr and Schnabel (*1921 c*), who found that herpes-immune rabbits resisted corneal and intracranial injection with encephalitis strains. Conversely, they found that animals recovered from a corneal infection with encephalitis strains were locally and generally immune to infection with herpes virus. Levaditi carried out similar experiments and was led to the same conclusions as regards the identity of the two types of strain (see Levaditi, Harvier, and Nicolau, *1922*).

Attempts were made to immunize rabbits actively by injections of encephalitis strains (Levaditi and Harvier, *1920 e*), and it was found that subcutaneous injections of fresh, etherized, or desiccated virus produced partial immunity as tested by the nasal or intra-ocular routes. It was not possible, apparently, to immunize sufficiently adequately to prevent encephalitis developing when the test injection was given intracerebrally. Guinea-pigs were immunized by repeated intraperitoneal injections of virus (Olitsky and Long, *1928*). It was impossible to demonstrate virucidal antibodies in actively immunized rabbits, although the sera of immunized sheep showed complement fixation with rabbit brain antigen (Levaditi, Harvier, and Nicolau, *1922*).

A number of workers has tested the virucidal properties of the serum of acute, chronic, and convalescent cases of encephalitis lethargica for encephalitis strains of herpes, with poor or negative results. Thus, Levaditi and Harvier (*1920 c*) found these antibodies present in the sera of only *1* out of *6* convalescents. Further, the neutralizing power was only apparent when the virus-serum mixture was injected corneally and not intracerebrally (Levaditi and Harvier, *1920 b*, Levaditi, Harvier, and Nicolau, *1921 a*). Blanc, Tsiminakis, and Caminopetros (*1921*) did not find any antibodies in the sera of convalescents of one year's standing.

It should be recalled that Andrewes and Carmichael (*1930*) and others (see Ch. XVII) have shown that the sera of about 75 per cent of normal persons have virucidal properties for known herpetic strains. Gay and Holden (*1931*), working in the same connection, showed that these properties were absent from the sera of cases of encephalitis, this confirmed the work just recorded in which workers failed to find any substantial evidence of virus neutralizing powers for encephalitis strains in the sera of convalescents.

It is claimed that herpetic eruptions are extremely rare in encephalitis lethargica (Netter, *1921 b*). This would scarcely seem to indicate an immune state, however, for it was found perfectly possible to produce herpetic vesicles in encephalitics by scarification of the arm and application of herpes virus thereto (Teissier, Gastinel, and Reilly, *1923*).

(D) A SUMMARY OF THE PRESENT POSITION

As will have become evident from consideration of the various properties of the strains isolated from cases of encephalitis, there is no doubt that they are true strains of herpes virus, as was first suggested by Blanc (*1921*). Accordingly, Levaditi and his coworkers claim that herpes virus causes encephalitis lethargica. Although this theory has never been accepted wholeheartedly outside France and Belgium, other workers in Britain (Da Fano, *1923*, *1924*, Perdrau, *1925 a*), in Switzerland (Doerr and his associates), and in the United States (Gay and Holden, *1932–3*, *1933*), have expressed opinions in its favor. Levaditi's theory will be critically examined in the following paragraphs.

1. Distribution of herpes virus in man (see Ch. XVII). The herpes virus is known to have a wide distribution, even in normal persons, for its presence has been recorded in the cerebrospinal fluid, saliva, and conjunctival secretion. Therefore,

on *a priori* grounds no virus obtained from the nasopharynx of encephalitic patients can be seriously considered as the etiological agent The great majority of encephalitis strains of virus has been isolated from the brain, but even here it is possible that

[illegible]

Only on about 20 occasions has herpes virus been isolated from cases of presumed encephalitis lethargica The theories brought forward in explanation have already been discussed. The theory of "autosterilizable neuro-infections" rests on no very strong basis of experimental evidence At any rate, it would not explain the absence of virus from the brains of chronic encephalitics, where the whole pathological process indicates the continued activity of the virus The demonstration, by Perdrau and others, that glycerol treatment may be needed to unmask a latent virus may certainly explain a number of failures to isolate virus from fresh brain. Yet if herpes virus is the cause of encephalitis lethargica, then it is almost unique in the difficulty with which it can be isolated In the case of rabies, poliomyelitis, and St Louis encephalitis, no difficulty is experienced in isolating by animal injection the characteristic causal agent. Moreover, in herpetic eruptions the virus is readily obtainable and no theories have had to be adduced to explain failures. The results of attempts to isolate herpes virus by animal experiments must, therefore, be regarded as very unsatisfactory

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4 *Pathological observations* Undoubtedly pathological observations do much to support herpes virus as the etiological agent of encephalitis There is considerable similarity between the pathological picture of acute encephalitis in the human being (see above) and that observed in the rabbit injected intracranially with herpes virus The main tissue changes are of the same order, and the "minute bodies" or inclusions of Da Fano are found in both (see Da Fano, 1923). Intranuclear inclusions occur in herpetic encephalitis and have been described in lethargic encephalitis. There is, however, even more histological similarity between human brains and those of partially immunized rabbits suffering from herpetic encephalitis (Perdrau, 1925 *a*) Herpetic infection of monkeys also resembles human encephalitis very markedly both clinically and histologically

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Levaditi has stated that 80 per cent of persons carry a keratogenic salivary virus and 15 per cent an encephalitogenic virus, both being typical strains of herpes.

Although these high figures have not been generally accepted, it is agreed that the saliva may on occasions contain herpes virus.

Levaditi's conception (Levaditi, Harvier, and Nicolau, 1921 *e*) of the etiology of encephalitis lethargica is that the virus exists in the saliva in a more or less attenuated form in between epidemics. During epidemics, either on account of a sudden increase in virulence of the virus, or on account of an increased susceptibility of the host, or of both, generalization occurs and encephalitis results. Unfortunately this conception is not based on any considerable amount of experimental work actually referable to man, and we cannot regard it as other than a possible theory

Conclusions.

It has been shown that encephalitis lethargica bears many of the characteristic stamps of a virus infection although no specific virus has yet been generally accepted as the causal agent.

There is no question that the virus of herpes febrilis can cause an encephalitis that resembles encephalitis lethargica in many ways The question that cannot yet be answered is whether herpetic encephalitis and lethargic encephalitis are identical, or whether the etiology of the latter condition awaits discovery. On the whole, the evidence of the last few years has tended to strengthen the view that herpes virus is the cause of lethargic encephalitis, although the position is far from clarified

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Although these high figures have not been generally accepted, it is agreed that the saliva may on occasions contain herpes virus.

Levaditi's conception (Levaditi, Harvier, and Nicolau, 1921 *c*) of the etiology of encephalitis lethargica is that the virus exists in the saliva in a more or less attenuated form in between epidemics During epidemics, either on account of a sudden increase in virulence of the virus, or on account of an increased susceptibility of the host, or of both, generalization occurs and encephalitis results. Unfortunately this conception is not based on any considerable amount of experimental work actually referable to man, and we cannot regard it as other than a possible theory.

Conclusions.

It has been shown that encephalitis lethargica bears many of the characteristic stamps of a virus infection although no specific virus has yet been generally accepted as the causal agent.

There is no question that the virus of herpes febrilis can cause an encephalitis that resembles encephalitis lethargica in many ways. The question that cannot yet be answered is whether herpetic encephalitis and lethargic encephalitis are identical, or whether the etiology of the latter condition awaits discovery. On the whole, the evidence of the last few years has tended to strengthen the view that herpes virus is the cause of lethargic encephalitis, although the position is far from clarified.

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Mild or abortive cases showed fever, headache, and systemic disturbances, but

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. were unable to resume their previous work. They found parkinsonism rare. The usual sequelae were headaches, irritability, some loss of memory, and drowsiness.

Cerebrospinal fluid. The fluid was clear and under slightly increased pressure. The cells averaged 50 to 250 per c mm, usually mononuclears, although the earliest counts might show up to 50 per cent polynuclears The sugar was normal, or

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12,000 to 20,000 per c mm,

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ly more commonly found.

Case fatality. The rate for St Louis (1933) was 20 per cent, but the rate for the oldest age group was double that of any other group. In the 1937 epidemic, the rate was 23 7 per cent. The Paris rate (1932) was 37 per cent. In fatal cases, death usually occurred after 6 days, and in over 50 per cent. within the first 2 weeks.

Pathology

The first full description of the pathological features was that of McCordock, Collier, and Gray (1934), founded on some 63 postmortems (see also Weil, 1934). The changes were almost entirely restricted to the central nervous system.

Brain and cord. Naked-eye, there was obvious vascular congestion, the cut surface being pink, and, in severe cases, the gray matter of the entire cortex, midbrain, pons, and cord was so affected In the usual case, however, the congestion was

tches in the cortex, basal nuclei, brain stem,

be found in the pia-mater in severe cases.

anges were fairly uniformly distributed throughout the central nervous system

1 The meningeal and intracerebral blood vessels were congested, with occasional perivascular hemorrhages Petechiae might be found in the brain parenchyma
2 Perivascular cuffing with lymphocytes was a regular feature.
3. Focal or diffuse infiltrations of microglia, plasma cells, and polynuclears were present Where this was a prominent feature, there was also neuronophagia
4 The extent of degeneration of nerve cells varied with the severity of the infection In mild cases, the changes were of the slightest, but in severe cases

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6 Very rarely small abscesses might be found in the parenchyma.

Other organs. In one-third of cases there was swelling and congestion of the

those of lethargic en-

Epidemiology

The first cases of "St Louis" encephalitis actually occurred in the summer of 1932 in Paris, Illinois (see Leake, Musson, and Chope, 1934). St Louis was not involved till the summer and fall of 1933 (1,100 cases) Then the disease spread to Kansas City and other parts within a radius of approximately 250 miles (Leake, 1933) In 1934 a similar outbreak occurred in Toledo, Ohio (Lowenberg and Zbinden, 1936) Outside this radius, however, serum neutralization tests showed New York to have been affected in 1933 (Webster, Fite, and Clow, 1935). In 1935 a

ST. LOUIS ENCEPHALITIS

In 1933 a remarkable outbreak of encephalitis occurred in St. Louis, and although other cities were visited, the disease has come to be known as St. Louis encephalitis It was realized that the disease was a specific entity unassociated with lethargic or other form of encephalitis then known in America. It closely resembled both in clinical and other respects Japanese (type B) encephalitis, but the viruses were immunologically distinct The outbreak was thoroughly investigated, and fully described in a *Bulletin* of the U.S. Public Health Service (*Report*, 1935).

A virus with characteristic action on monkeys and mice was isolated and has been extensively studied It was not, however, possible to isolate this virus from all cases of apparently typical St. Louis encephalitis (McKinley and Verder, 1933-4) There was a recurrence of the disease in the summer of 1937 (see *J. Amer med Ass*, 1937, Hempelmann, 1938), and since this date the virus has spread widely in the United States It has been studied particularly by Hammon and associates on the West Coast

FEATURES OF 1933 AND 1937 OUTBREAKS

Clinical Features

The symptoms were first described in detail by Leake (1933) and Hempelmann (1934) The features of the 1937 recurrence were similar (see Hempelmann, 1938)

Incubation period This was ascertained accurately in persons entering the infected zone who developed encephalitis within 9 to 21 days of arrival Probably the outside limits were 4 to 21 days

The onset was sudden with headache, high fever, neck rigidity, mental confusion, tremors, and only transient ocular manifestations.

The encephalitic type was characterized mainly by symptoms referable to the central nervous system, and the features were unquestionably those of an encephalitis. The height of the disease was reached in 24 to 48 hours The neck and spine were rigid, Kernig's sign positive, and hyperesthesia was noted, the tendon and plantar reflexes were variable, and the abdominal absent. The pupils were usually small and equal There was considerable mental impairment with some disorientation for time and space Although drowsy and apathetic, certain patients could usually be roused, other patients being sleepless or wildly delirious At the height of the disease, speech was often slurred or aphasia present Fine tremors involving the hands, tongue, and lips sometimes lasted for 2 to 3 weeks

As regards vision, there was sometimes blurring, but severer manifestations such as ptosis were very rare About 25 per cent showed vertigo, ataxia was rarer, and only a few patients showed facial paralysis or spastic monoplegias Involvement of the cord was suggested by the frequency with which retention or incontinence of urine occurred

Fever was usually present, being highest for the first 2 or 3 days (up to 105° F) and falling by lysis to normal in 7 to 10 days, occasionally, however, it might be prolonged irregularly for a month or more

The general infection type showed no specific evidence of involvement of the central nervous system until a few days after the onset. During this period there were symptoms of chills, rigors, neuromuscular pains, headache, photophobia, sore throat, and pyrexia Then the temperature rose, and the characteristic encephalitic picture became manifest.

Mild or abortive cases showed fever, headache, and systemic disturbances, but the nervous manifestations were slight or absent.

After history. Bredeck *et al.* (1938) have recorded a "follow up" study of over 300 sufferers from the original (1933) epidemic. They found that 66 per cent. had completely recovered Only 6 3 per cent. were unable to resume their previous work. They found parkinsonism rare. The usual sequelae were headaches, irritability, some loss of memory, and drowsiness.

Cerebrospinal fluid The fluid was clear and under slightly increased pressure. The cells averaged 50 to 250 per c mm, usually mononuclears, although the earliest counts might show up to 50 per cent. polynuclears The sugar was normal, or slightly increased, and the globulin moderately increased.

The blood occasionally showed a white cell count of 12,000 to 20,000 per c.mm, with a shift to the left of the Schilling count, but these findings were unassociated with the severity of the illness Leukopenia was probably more commonly found

Case fatality. The rate for St. Louis (1933) was 20 per cent., but the rate for the oldest age group was double that of any other group. In the 1937 epidemic, the rate was 23 7 per cent. The Paris rate (1932) was 37 per cent. In fatal cases, death usually occurred after 6 days, and in over 50 per cent within the first 2 weeks.

Pathology

The first full description of the pathological [illegible] McCordock, Collier, and Gray (19[illegible]) [illegible] [illegible]eil, 1934). The changes [illegible]

Brain and [illegible] [illegible]ascular congestion, the cut surface being pin[illegible] [illegible] cases, the gray matter of the entire cortex, midbrain, pons, and cord was so affected In the usual case, however, the congestion was more blotchy, occurring in scattered patches in the cortex, basal nuclei, brain stem, and cord. Petechial hemorrhages might be found in the pia-mater in severe cases.

Microscopically, the following changes were fairly uniformly distributed throughout the central nervous system

1 The meningeal and intracerebral blood vessels were congested, with occasional perivascular hemorrhages Petechiae might be found in the brain parenchyma.
2 Perivascular cuffing with lymphocytes was a regular feature
3 Focal or diffuse infiltrations of microglia, plasma cells, and polynuclears were present Where this was a prominent feature, there was also neuronophagia
4 The extent of degeneration of nerve cells varied with the severity of the infection In mild cases, the changes were of the slightest, but in severe cases there was great shrinking of the cell body, pyknosis, or even complete disappearance No inclusions could be found in nerve cells
5 The meningeal reaction was lymphocytic, never purulent, and was most intense basally.
6 Very rarely small abscesses might be found in the parenchyma

Other organs In one-third of cases there was swelling and congestion of the kidneys with hemorrhagic pyelitis.

It is evident that these pathological features differ from those of lethargic encephalitis, particularly in their more widespread distribution

Epidemiology

The first cases of "St Louis" encephalitis actually occurred in the summer of 1932 in Paris, Illinois (see Leake, Musson, and Chope, 1934) St Louis was not involved till the summer and fall of 1933 (1,100 cases) Then the disease spread to Kansas City and other parts within a radius of approximately 250 miles (Leake, 1933) In 1934 a similar outbreak occurred in Toledo, Ohio (Lowenberg and Zbinden, 1936) Outside this radius, however, serum neutralization tests showed New York to have been affected in 1933 (Webster, Fite, and Clow, 1935). In 1935 a

somewhat similar outbreak occurred in Windber, Pa (Slesinger, 1936). The disease seemed to have disappeared after these outbreaks, as no virus could be isolated from various cases of encephalitis between 1933 and 1937 (McCordock, Smith, and Moore, 1937–8).

In 1937, however, another summer outbreak occurred in St. Louis city and county. Clinically the outbreak resembled the earlier in every respect. Between June 30 and October 18 there were 412 cases (see *J. Amer. med. Ass.*, 1937, Zentay and Basman, 1939). The disease must now be considered as endemic in the United States.

All the outbreaks occurred in the summer and fall, and were strictly limited in duration. Throughout the St. Louis (1933) epidemic, more cases were reported from the suburban than from the city areas, and in the early stages the country rate was 11 times that of the city rate. There was a remarkable freedom from multiple cases in families in all the outbreaks except in Windber, where whole families were involved Once the disease was established, the susceptibility of the individual was more important than exposure to contagion, thus it was often the member of the house least able to move about who was affected

There was no racial, sex, or class predilection, but there was a striking increase in the incidence of the disease with advancing age (Leake, 1933). Thus, 13 per cent of cases occurred in the 25 per cent. of population under 15, 23 per cent. of cases in those from 15 to 34, 29 per cent. of cases in those from 35 to 54, and 35 per cent in those over 55 (only 13 per cent. of the population). In the Windber outbreak, however, only one-tenth of the cases were over 30 years.

It was shown that cases, both in 1933 and 1937, appeared to occur in the vicinity of small streams, in areas characterized by weeds, refuse dumps, open sewage, and ponds, suggesting the rôle of insects (Casey and Brown, 1938, Casey, 1942).

Drinking water and milk were both excluded as sources of infection, and as epidemics occurred in summer, suspicion naturally fell on insect vectors Mosquitoes were considered to be the only likely insects with a range wide enough to explain the distribution of cases. Experiments were carried out with *Aedes aegypti*, *Anopheles quadrimaculatus*, and *Culex pipiens* Although the insects undoubtedly absorbed virus which persisted till their death, they did not infect men, mice, or monkeys by biting (Leake, Musson, and Chope, 1934, Webster, Clow, and Bauer, 1935) The method of spread was not solved, although suspicion rested on insect vectors

On the basis of experimental work, it was tentatively suggested that mice might constitute a reservoir of infection (Harford *et al.*, 1939 *a*, *b*).

In the St. Louis outbreak the health authorities did not insist on quarantine, but isolation was carried out in general (not fever) hospitals (Bredeck and Zentay, 1934)

Rôle of Streptococci

Rosenow has claimed a primary etiological rôle for streptococci (Rosenow, 1933, 1933–4, Rosenow and Jensen, 1933).

FEATURES OF LATER OUTBREAKS

The clinical features of later outbreaks seem similar to those originally described in St. Louis.

It is now recognized that abortive cases may occur Thus Blattner and Heys (1946) isolated virus from the blood of a child suffering from drowsiness, fever, and vomiting St Louis infection must be considered in the differential diagnosis of nonparalytic poliomyelitis and equine encephalitis. Infections due to two of these agents have occurred simultaneously in the one locality.

St. Louis District

From 1939–1944, 12 cases of St. Louis encephalitis were investigated at the Children's Hospital, and it appears that the disease is now endemic in the area (Blattner and Heys, 1945).

California

[illegible] osis being [illegible] ley, 1939, [illegible] must be considered endemic (Howitt, 1942; Hammon, Reeves, and Galindo, 1945). There were 6 cases in 1943, and 3 in 1944 confirmed by isolation of the virus (Hammon and Reeves, 1947). Virus has been isolated from the brain of a fatal case (Lennette, 1946). Most of the cases have occurred in the rural areas of the San Joaquin Valley (especially Kern County).

Antibodies have been found in the sera of animals in the endemic areas, especially horses and birds, such as fowls, antibodies have frequently been found to St. Louis and western equine encephalitis in the one sample; the sera of a few wild rodents have also been found positive (California Mosquito Control Association, 1942, Howitt and van Herick, 1941, 1942, Hammon, Reeves, and Galindo, 1945; Hammon and Reeves, 1947).

There is little doubt that the infection is spread by mosquitoes, and virus has been recovered from *Aedes dorsalis* (Hammon and Reeves, 1945, 1947).

Yakima Valley, Washington

Hammon and his associates made extensive studies on the occurrence of western equine encephalitis and St. Louis encephalitis in man and horses in this valley in the State of Washington, especially from 1939–1942 (see also Ch XCVI). Epidemics occurred each year in July and reached their peak in mid-August; 81 per cent of cases were males, and the case fatality was 22.4 per cent (Hammon, 1941, Hammon and Howitt, 1942)

St Louis infection is endemic in this area, and the sera of many "normal" residents neutralize the virus, the percentage showing antibodies increases with time of residence in the area (Hammon, 1943)

The disease is spread by mosquitoes, and St Louis virus has been isolated from *Culex tarsalis* on 8 occasions, the infection rate being at least 1 210, *Culex pipiens* has also been found infected, there is little doubt that *Culex tarsalis* is the main vector of St Louis and western equine viruses in Yakima (California Mosquito Control Association, 1942, Hammon *et al*, 1942 *b*, *c*, 1945, Hammon and Reeves, 1942, 1945, Reeves and Hammon, 1942) A later report (Hammon and Reeves, 1947) refers to further isolations of virus from *C tarsalis*.

Culex tarsalis has been found to feed on fowls, domestic animals, and man, *Culex pipiens* feeds exclusively on fowls (Reeves and Hammon, 1944). This finding is of importance, and it appears that chickens may constitute an important reservoir of infection, although a much lower percentage of their sera neutralized St Louis than western equine encephalitis virus (Hammon, 1941, Hammon *et al*, 1941 *a*, 1942 *a*, 1945). There is not yet any evidence to incriminate chicken mites in the spread of the virus between chickens in this area (Hammon and Reeves, 1947) The sera of horses and other domestic animals, and a few wild mammals, also neutralized St. Louis virus (California Mosquito Control Association, 1942; Hammon, 1941, Hammon and Howitt, 1942, Hammon *et al*, 1941 *a*, 1942 *a*).

Other Areas in America

Arizona An epidemic occurred in Pinal County among migratory laborers, involving about a dozen people, half being children (Meiklejohn and Hammon, 1942, Hammon, 1943)

Colorado. There was an epidemic of encephalitis in man and horses in 1940 in N. Colorado. Serum samples neutralized the St. Louis virus (Philip, Cox, and Fountain, 1941). Studying the mosquito population in 1943 and 1944, Black, Absher, and McDonald (1945) found *Culex tarsalis, C. pipiens,* and *Mansonia perturbans* to be present.

Minnesota A few cases may have occurred in 1941 (Eklund, 1946).

Oklahoma. Antibodies to the St. Louis virus have been found in the sera of some mammals, but not in chickens (Reeves, Mack, and Hammon, 1947).

Texas. Studies were carried out in Texas in 1942 by Hammon, Reeves, and Irons (1944). Men and horses had probably been infected the year previously with St. Louis (and western equine) virus. Antibodies to St. Louis virus were detected in the sera of fowls and some domestic animals.

PRESENT KNOWLEDGE OF THE EPIDEMIOLOGY

The Rôle of Mosquitoes and Other Arthropods

Mosquitoes.

In the earlier outbreaks, evidence was strong that the disease was spread by mosquitoes, but there was no definite proof Of recent years, however, the work of Hammon and his associates has shown conclusively that mosquitoes, mainly *Culex tarsalis*, are the responsible vectors in Yakima, Washington, and probably elsewhere

Thus, mosquitoes have been found infected in endemic areas, for virus has been recovered from *Culex tarsalis* and *Culex pipiens* obtained in the Yakima Valley, *C tarsalis* is the main vector (Hammon *et al*, 1941 *b*, 1942 *b, c*, 1945, California Mosquito Control Association, 1942, Hammon and Reeves, 1942, 1945, 1947, Reeves and Hammon, 1942). Virus has also been recovered, in California, from *Aedes dorsalis* (Hammon and Reeves, 1945, 1947). The necessary technique has been described by Hammon, Reeves, and Izumi (1942).

The following mosquitoes have been shown capable of transmitting the infection by bite, in laboratory experiments (Hammon and Reeves, 1942, 1943; Hammon, Reeves, and Gray, 1943, Reeves, Hammon, and Izumi, 1942, see also Fulton *et al*, 1940)· *Culex tarsalis, Culex pipiens, Culex coronator, Aedes lateralis, Aedes taeniorhynchus, Aedes vexans, Aedes nigromaculis, Theobaldia incidens, Theobaldia inornata.*

Hammon and Reeves (1945) quote work of Mitamura in which infection was transmitted by *Culex tritaeniorhynchus, Aedes albopictus,* and *Culex pipiens* var *pallens.*

In these experiments, mosquitoes have been infected by a meal of virus-containing material or by biting an infected animal After a few days the infection has been transmitted by bite to chickens, pigeons, doves, and baby mice. Of particular interest is the finding that *Culex tarsalis* can be infected by feeding on chickens and ducks previously inoculated with virus subcutaneously and developing viremia, after a period these mosquitoes transmitted the infection to other chickens, and these developed viremia (Hammon and Reeves, 1943).

C. tarsalis feeds in nature on cows, horses, men, pigs, dogs, chickens, and sheep, and is, therefore, a potent danger as regards the transmission of infection (Bang and Reeves, 1942).

Ticks.

St. Louis virus has been recovered from engorged larval ticks fed on infected Swiss mice, experimentally, infection has been transmitted to young mice by larval ticks (Blattner and Heys, 1941) In later experiments (1943, 1944), it was reported that the female could transmit the infection to her offspring through the egg, and through all stages of metamorphosis to the second generation. It was suggested that

ticks may maintain the infection in lower animals, but that they are not responsible for human infections.

Mites.

St Louis virus has been isolated from chicken mites (*Dermanyssus gallinae*) obtained in the St Louis area, on 7 separate occasions, in nonepidemic periods, mites naturally infected retained virus after 5 months in the laboratory, transfer through the egg to the first stage nymph has been shown (Smith, Blattner, and Heys, 1944, 1945). In later experiments, it was shown that the small amount of virus circulating in the blood of chickens was sufficient to infect fresh mites (1947). Hammon and Reeves (1947) found no evidence that mites were infected in Yakima.

Animal reservoirs.

It has been found that the sera of horses and other domestic animals may contain antibodies to the virus, these antibodies may also be found in the sera of chickens, antibodies have less commonly been found in the sera of wild birds and mammals (Hammon *et al*, 1941 *a*, Hammon, Carle, and Izumi, 1942).

As has been mentioned above, the most important reservoir is probably the chicken, as it develops viremia, and can infect mosquitoes that bite it; other domestic birds may also be responsible.

Conclusions.

Although it is reasonable to postulate the spread of the disease by mosquitoes to fowls, with occasional and accidental involvement of horses and man, the story is far from complete (Hammon and Reeves, 1945) There have been no positive isolations of virus from overwintered mosquitoes, and there is no evidence of transovarial spread It would seem that the demonstration of infection in chicken mites is of considerable importance, although Hammon and Reeves (1945) failed to isolate virus from over 20,000 mites from Yakima in 1944

ANTIGENIC CHARACTERISTICS OF ST. LOUIS VIRUS

The strains isolated in 1933 by numerous workers have been passaged continuously since that date Various strains of virus were isolated from the 1937 outbreak (Greutter *et al*, 1937–8, McCordock *et al*, 1937–8)

Strains isolated in St Louis in 1933 and 1937, Kansas City, Paris, and New York were shown to be immunologically identical (Webster and Fite, 1933, 1933–4, 1935, Greutter *et al*, 1937–8, McCordock *et al*, 1937–8). However, Webster (1940) referred to slight differences between 2 strains

ANIMAL EXPERIMENTS

Mice.

Laboratory mice are highly susceptible to infection, field mice can also be infected (Greutter *et al*, 1940) Young mice are more susceptible to peritoneal inoculation than are older animals, but when virus is inoculated cerebrally or nasally there is no difference in susceptibility depending on age (O'Leary, Smith, and Reames, 1942)

Intracranial injection is the simplest method of transmitting the infection As first shown by Webster and Fite (1933), white-face, Swiss, and other mice are susceptible The virus multiplies rapidly in the brain and reaches a titer of 10^9 intracerebral lethal doses After an incubation period of 3 to 4 days, the fur becomes ruffled and the skin hyperesthetic. Tremors, convulsions, ataxia, paralysis, and prostration follow, and the animal dies within 5 to 9 days after injection (Webster and Fite, 1933, Muckenfuss, Armstrong, and McCordock, 1933, Brodie, 1933–4 *b*)

The pathological features are similar whether the encephalitis follows intracerebral, intranasal, or other route of injection (Webster and Fite, 1933; Smadel and Moore, 1934). Naked-eye, the brain is pink. Microscopically the following changes are found, (*a*) perivascular cuffing with mononuclears, (*b*) focal collections of microglia related to vessels unaffected by cuffing, (*c*) a combination of (*a*) and (*b*), (*d*) degenerative changes in the ganglion cells, chromatolysis, pyknosis, loss of Nissl granules, or neuronophagia, (*e*) infiltration of the meninges and choroid plexuses with mononuclears, (*f*) occasional perivascular hemorrhages; (*g*) perivasc [illegible]
cor[illegible]
are [illegible]
the summer (Lillie *et al.*, 1937).

Intranasal inoculation. Mice can be infected readily by intranasal instillation of virus (Webster and Fite, 1933; Brodie, 1934–5, and many others), and the presence of the virus can be demonstrated in the olfactory bulbs in 24 hours, the pyriform lobes in 24 to 48 hours, the remainder of the brain in 3 days, and the cord in 4 days (Webster and Fite, 1934 *a, b*, Brodie, 1934–5, Webster and Clow, 1936)

Microscopic lesions in the brain follow the arrival of the virus Thus not until the 3rd day do lesions appear in the olfactory bulbs, the 4th day in the pyriform lobes, and the 5th day in Ammon's horn. These lesions take the form of perivascular exudation of mononuclears in the pia, hyperplasia of the pial endothelium, and necrosis of the nerve cells of the olfactory tract (Webster and Clow, 1936) Rake (1937) confirmed the passage of virus to the forebrain after intranasal instillation, this did not occur before 24 hours. This time was much more than that obtained with equine encephalomyelitis virus. Cook (1938 *a*) reported that neither various bacteria nor the viruses of human and swine influenza altered the pathogenicity of St. Louis virus for mice, when sprayed together in the form of a mixture intranasally Occasionally, subclinical infection may develop after nasal inoculation (Slavin, 1943).

Other methods of injection Encephalitis may ensue, especially in young mice, after intraperitoneal or subcutaneous injection, in older animals large doses may infect (Webster and Clow, 1936, Lennette and Koprowski, 1944).

Other routes that have been used include vaginal application (Haber, 1939 *b*), ingestion (Harford and Bronfenbrenner, 1942), or intestinal inoculation (Mezera *et al*, 1941), intratesticular (Lennette and Smith, 1939 *a*, 1940), intramuscular, when glycerol is given peritoneally (King, 1940), intravenous (Peck and Sabin, 1947)

Effects of breeding Webster (1937) selected from a hybrid stock (in which 40 to 50 per cent died after injection of virus) lines in which as high as 95 per cent or as low as 15 per cent died after injection of the virus He suggested that the resistance to St Louis virus is inherited on a single factor basis with resistance dominant over susceptibility (see also Wright, 1940).

Pathogenesis and distribution of virus

When virus is inoculated by peripheral routes, it seems certain that it invades by nerve paths to reach the brain and cord, and cause encephalitis and myelitis, the virus may ascend directly or perhaps by initial blood invasion and excretion into the nasal mucosa (Holden, 1940, O'Leary, Smith, and Reames, 1942)

Virus may be found in the blood shortly after injection, and just before death, in the spleen, and less commonly in other organs (Webster and Fite, 1934 *b*, Webster and Clow, 1936, Kudo *et al*, 1937, Haber, 1938, 1939 *a*) A special study of the dissemination of virus in the body has been made by Peck and Sabin (1947)

1 After intravenous inoculation of large doses, they could only recover at the most about 1/1,000 of the inoculum 4 hours later, and this from the blood, spleen, liver, lungs, and heart. At 24 hours, traces were found in the brain, intestinal tract, tongue, nasal mucosa, hamstring muscles, and inguinal lymph nodes At 48 hours,

72 hours
ct It ap-
eripheral
nerves, as it was found in sciatic nerves, and hamstring muscles. At 96 hours, when the animal was prostrate and the virus concentration in the brain very large, virus was disseminated throughout the body, but the blood and nasal mucosa had only traces. It appeared that virus multiplied primarily only in the CNS, and to a lesser extent in the intestinal tract.

us injection of large
in the CNS Occa-
was primary local-
e posterior extremi-
cur in the lumbar
region, as seen after injection in the muscles of the posterior extremities

2. After intracerebral injection of at least 100 cerebral LD50, no virus could be detected locally after 4 hours. At 24 hours there was evidence of early multiplication in the brain, and a trace of virus was found in the spleen At 48 hours, 4 million LD50 were found in the brain, and centrifugal spread along peripheral nerves was beginning. At 96 hours, the virus concentration in the brain was 3.2 billion LD50, and virus was found in all organs except inguinal nodes and intestinal contents. Virus had evidently "spilled over" into the blood

3 After nasal inoculation, virus quickly reached the lungs, but did not undergo continued multiplication there, at 48 hours there was evidence of commencing multiplication in the brain At 96 hours, the distribution of virus resembled that found in the cerebral group There was no evidence of multiplication in the nasal mucosa.

4 Tests indicated that virus could be present in high concentration in the walls of the intestinal tract, without being liberated in the stools

5 In the terminal stages of the infection, leg muscles, heart, lungs, spleen, tongue, and urinary bladder contained as much virus as, or more than, the sciatic nerves, per unit of weight. As the amount of virus in the blood was negligible, it seems that virus either accumulated in these tissues by diffusion along nerve fibers, or multiplied in some other constituent than nerve fibers.

Prevention or modification of experimental infection of mice.

(*a*) *By inoculation of chemicals* Intranasal inoculations of picric acid, with or without alum, were found to be the most effective means of preventing infection by the same route. Picric acid is thought to act locally on the mucosa, on the virus itself, or on both (Armstrong and Harrison, 1936 *a*) It was found that picric acid solutions have no protective effect unless the pH is such that coagulation of proteins occurs (Armstrong and Harrison, 1936 *b*) Saline (4 per cent) and hypertonic glucose have a similar though less pronounced effect (Armstrong, 1935). Injections of prontosil and related drugs were ineffective (McKinley, Meck and Acree, 1939). Mice showing signs of infection are more susceptible than normal animals to the lethal effects of intraperitoneally injected glucose, due to a dehydrating effect (Hoyt, Holden, and Rawson, 1939).

(*b*) *By x-rays* Mice treated with soft x-rays after intranasal instillation of virus either fail to succumb, or else the incubation and duration periods of the disease are prolonged (Goldberg, Brodie, and Stanley, 1934–5)

(*c*) *Cultures of nonpathogenic bacteria* were found to render mice resistant to intracranial inoculation, the cultures were obtained from the nostrils of mice and were injected intranasally in the test animals (Armstrong, 1938)

(*d*) *An interference phenomenon* has been demonstrated, for a cerebral inoculation of influenza virus interferes with the proliferation of St. Louis virus in the brain (Vilches and Hirst, 1947)

Monkeys.

Tremors, hyperirritability, raised temperature, pareses, apathy, and drowsiness follow the intracranial injection (repeated if necessary) of rhesus monkeys in 8 to 14 days Pathologically the changes are perivascular cuffing, nerve cell degeneration, and focal cellular infiltrations The virus is not readily established in monkeys and dies out with passage, cebus monkeys are insusceptible altogether (Mucken- [illegible] 1933; Webster and Fite, 1933, [illegible] Webster, 1934). Viremia may [illegible]

During the course of passage through monkeys, Armstrong and Lillie (1934) encountered a virus which they named the virus of experimental lymphocytic choriomeningitis (see Ch LXXXIX).

Other animals.

Rabbits are insusceptible to intracranial, corneal, intradermal, and intraperitoneal injections (Brodie, 1933–4 *b*, Webster and Fite, 1935).

Guinea-pigs were said to be insusceptible to intracranial and pad injections (Webster and Fite, 1935; Brodie, 1933–4 *b*), although Sulkin *et al* (1939 *b*) reported some positive results on intracerebral injection

Rats are insuspectible to intracranial, nasal, and intraperitoneal injections (Brodie, 1933–4 *b*, Webster and Fite, 1935). Smith (1939 *b*) reported that virus persisted in the brains of rats and guinea-pigs for 8 to 9 days after injection of virus.

Syrian hamsters can be infected (Lennette, 1941; Broun *et al*, 1941 *a*)

Sheep are insusceptible to intracranial injection (Webster and Fite, 1935).

Kittens and ferrets are insusceptible to injection (Brodie, 1933–4 *b*).

Chickens, ducks, and *doves* develop viremia after subcutaneous injection (Hammon, Reeves, and Izumi, 1946).

Horses can be infected by the cerebral route (Cox, Philip, and Kilpatrick, 1941).

Chicks (newly hatched) develop no definite signs, but virus may be passed in series for a time using brain tissue (Pearson, 1940, Sulkin, Harford, and Bronfenbrenner, 1940).

Morphology and Filtrability

The size has been estimated at from 0 22 μ to 0 33 μ by Elford's filtration method (Bauer, Fite, and Webster, 1933–4), and by Elford and Perdrau (1935) at from 0 20 μ to 0 30 μ (see also Yaoi *et al*, 1939).

As yet no inclusion bodies have been described.

The virus filters through Berkefeld V and N, and Seitz filters (Webster and Fite, 1933, 1935)

Tissue Cultivation

The virus has been grown in various types of tissue culture (Syverton and Berry, 1935, Harrison and Moore, 1936–37, 1937, Schultz *et al*, 1938, Molloy, 1940, Pang, 1940, Pang and Zia, 1940, Webster and Johnson, 1941, Huang, 1943 *a*, *b*)

The Egg

The virus proliferates in the egg after inoculation by the usual routes, the chorio-allantoic membrane being chiefly used (Harrison and Moore, 1936–7, 1937, Schultz *et al*, 1938, Smith, 1939 *a*, 1943 *b*, Smith and Lennette, 1939, Blattner and Heys, 1946, Smith, Blattner, and Heys, 1947)

The chorio-allantoic *route can be used for the titration* of viruses, and neutralizing antibodies (Blattner and Cooke, 1942).

It has been reported that western equine encephalitis virus grows only to a

limited extent when inoculated in eggs supporting the growth of St Louis virus (Duffy, 1944).

Reaction to Physical and Chemical Agents

Virus in the form of brain suspension rapidly loses infectivity in the ordinary ice chest. Infectivity is well maintained in vials in the dry ice chest. Glycerol is said to preserve infectivity for some months. Infectivity may be preserved for prolonged periods in frozen dried brain tissue (Lennette and Smith, 1939 *b*, Wooley, 1939)

Infectivity is not well preserved in saline, and serum or skimmed milk should be used (see, e g., Cook and Hudson, 1937). The optimum pH for stability (in glycine phosphate buffer) is 8 4–8 8 (Duffy, 1946).

As regards heat, infectivity is lost in half an hour at 56° C. (Kudo *et al.*, 1937).

Virus can be inactivated by exposure for a brief period to a suitable source of ultraviolet light, such preparations are antigenic (Levinson *et al*, 1944, 1945).

Soft x-rays also inactivate virus (Moore and Kersten, 1937).

Virus is inactivated by 0 1 per cent. formalin in 10–18 hours at room temperature, but resists 1 per cent phenol for 25 days, and ½ per cent for 50 days (Brodie, 1933–4 *b*)

The virus is sensitive to ether *in vitro*; ether anesthesia alters the course of infection with the virus, but this is unlikely to be due to a direct virucidal effect (Sulkin, Zarafonetis, and Goth, 1946, Sulkin and Zarafonetis, 1947)

Penicillin has not been shown to exert any virucidal effect in eggs or tissue cultures (Parker and Diefendorf, 1944). Numerous chemotherapeutic agents were tested by Kramer, Geer, and Szobel (1944), but none was shown to have any action

IMMUNITY

Active Immunity

It has been shown that mice can be actively immunized by intranasal inoculation (Armstrong, 1934, 1935) and subcutaneous and intraperitoneal injections (Webster and Fite, 1934 *a*, *b*; Webster, 1938, Hodes, 1939).

The immune state develops in 7 days or so, and is manifested by resistance to further injections of virus The immunity lasts for 4 to 6 weeks, and has disappeared [illegible] ially immune [illegible] (Armstrong, [illegible] reinoculated intracerebrally or nasally with virus 7 to 21 days after one or two previous injections [illegible] laccid [illegible] velop [illegible] days when the reinoculation was intracerebral, and after 11 to 14 days when intranasal Histologically the cords of paralyzed mice showed vascular congestion, perivascular and more diffuse round cell infiltration, with microgliosis and degeneration of nerve cells

Hodes (1939) reported that it was much easier actively to immunize virgin than pregnant mice Sulkin *et al* (1939 *a*) immunized mice with intranasal inoculation of nasopharyngeal [illegible]

The local use o [illegible] eveloping after nasal instillation of [illegible]

Virus propagate [illegible] properties for mice (Smith, 1943 *b*)

Sabin (1943) has shown that immunity to peripheral challenge can be produced in mice by the inoculation of formolized lyophilized brain suspension Virus in-

activated by ultraviolet light also induces a high degree of resistance (Levinson *et al.*, 1944, 1945).

Virus Neutralizing Antibodies

Antibodies that neutralize the infectivity of St. Louis virus develop in the sera of animals on recovery from infection, or after vaccination with live or inactivated virus.

Virus neutralizing antibodies are usually titrated by inoculation of virus-serum mixtures intracerebrally in mice, and standard procedures have been described by Hammon and Izumi (1942), and Olitsky and Casals (1947, see also Ch. XI). Antibodies can also be titrated in susceptible young mice, injections being subcutaneous in 3-day animals, and peritoneal in animals of 8–28 days, the younger the animal, the greater the amount of neutralization demonstrated (Lennette and Koprowski, 1944).

It has been found that the lipoid fraction of certain normal animal sera rapidly reduces the infectivity of St Louis virus (Casals and Olitsky, 1947)

The sera of certain Brazilian marsupials and rodents neutralizing yellow fever virus also inactivated one or more of the following, Japanese, St Louis, and West Nile viruses, not known to occur in S. America; the inactivating substance resisted 56° C. (Koprowski, 1946).

If pregnant mice are immunized, transmission of antibodies occurs, and this may give rise to a degree of resistance in the sucklings (Smith, 1943 *a*, Slavin, Hale, and Berry, 1946).

Antibodies develop in convalescence from the human disease, and are usually found after about 10–14 days, the antibody titer remains high for some years, antibody also develops on recovery from a subclinical attack (Barr, 1934, Wooley and Armstrong, 1934, Webster, Fite, and Clow, 1935, Muckenfuss, Smadel, and Moore, 1938, Smith and Moore, 1938, Howitt, 1943)

Maternal transmission of antibodies may occur in young children (Hammon *et al*, 1945).

Convalescent serum has been used in prophylaxis and treatment (Slesinger, 1936)

Complement Fixation

Complement fixation can be demonstrated between antisera and various types of [illegible] exposing to ultraviolet light, [illegible] Palacios, 1941, Casals, 1942, [illegible] *was imparted to* mouse brain or chick embryo antigens by extraction in the lyophilized state with benzene, toluene, or dichlorethylene (DeBoer and Cox, 1947; Espana and Hammon, 1947, Hammon and Espana, 1947)

Complement fixation can be used in the diagnosis of the disease in man (Howitt, 1943, Casals, 1945)

Miscellaneous Reactions

It has been reported that virus-coated bacteria are clumped by antisera (Roberts and Jones, 1941, 1942, Roberts, 1945)

Mechanism of Resistance

Various observations suggest that there is little correlation between the presence of serum antibodies and resistance to challenge Thus Webster (1938) found that at the time of maximum resistance to reinoculation, no antibodies could be demonstrated Hodes and Webster (1938) found that after vaccination, mice were resistant to cerebral challenge within one week, and that this resistance was at a high level for 6 weeks, then waning to disappear between the 12th and 20th weeks

They could not demonstrate virus neutralizing antibody until 8 weeks after vaccination. The antibodies were at their highest after 23 weeks, when the degree of resistance was low Observations of similar significance were made by Casals (1943) in mice, complement fixing antibodies developed equally well in those showing or not showing resistance.

Working with hamsters, Broun *et al* (1941 *b*) concluded that the presence of humoral antibodies did not insure resistance.

RELATIONSHIP TO OTHER VIRUS INFECTIONS

Japanese Encephalitis and West Nile Viruses

The virus of St. Louis is closely related to these 2 viruses, which form a subgroup in the group of neurotropic viruses

Certain differences can be detected between the behavior of St Louis and Japanese viruses Thus Kudo *et al.* (1937) concluded that. in mice injected cerebrally, paresis is commoner with the Japanese virus; after intraperitoneal or subcutaneous injection, St Louis virus rarely produces encephalitis, but this is more common with Japanese virus, monkeys are more susceptible to the Japanese than the St. Louis virus

Antigenically Japanese and St Louis viruses are closely related The Japanese [illegible] protective power [illegible] the Japanese virus [illegible]). [illegible] West Nile virus, [illegible] here that in active [illegible] between the three agents (Smithburn, 1942, Casals, 1944). In neutralization tests, definite cross reactions occur, both St. Louis and Japanese sera neutralizing the West Nile virus (Smithburn and Jacobs, 1942, Casals, 1944) West Nile sera only neutralize the other two viruses if of high titer (Casals, 1944).

There seems little doubt that the two agents share common antigens, and that Japanese virus is the most, and West Nile the least, complete of the three, St Louis virus occupying an intermediate position (see also Lennette and Koprowski, 1946)

Encephalitis Lethargica

The isolation of the characteristic virus from cases of St Louis encephalitis renders any relationship with encephalitis lethargica unlikely In confirmation of this, the sera of a number of acute encephalitic and of parkinsonian cases showed no virus neutralizing powers for St Louis virus (Brodie, 1933–4 *a*, Webster, Fite, and Clow, 1935).

Other Viruses

No relationship has been disclosed by neutralization or complement fixation tests with herpes, poliomyelitis, lymphocytic choriomeningitis, rabies, equine encephalomyelitis, vesicular stomatitis, louping ill, blue tongue, and fox encephalitis (Cox and Fite, 1933–4, Webster and Fite, 1933, 1933–4, Webster, Fite, and Clow, 1935, Howitt, 1937)

According to Casals (1944) the West Nile, Japanese, and St Louis viruses are unrelated to the viruses of spring-summer encephalitis, louping ill, or western equine encephalomyelitis Russian observers, however, claim that Russian and louping ill viruses are related antigenically to Japanese and St. Louis viruses (see Ch. XCVIII) The group is not related antigenically to Bunyamwera virus (Smithburn, Haddow, and Mahaffy, 1946), Anopheles A virus (Roca-García, 1944), or Colorado tick fever virus (Koprowski and Cox, 1947).

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CHAPTER XCIV

JAPANESE TYPE B ENCEPHALITIS

Two types of epidemic encephalitis occur in Japan, the ordinary or von Economo's encephalitis lethargica (type A), and "summer" or type B encephalitis (Kaneko and Aoki, 1928).

Type A differs in no important essential from the description of encephalitis lethargica given above (Ch. XCII). Although cases of Type B or summer encephalitis have probably been recognized in Japan since the middle of last century, it is only comparatively recently that any serious bacteriological investigations have been made. Numerous workers have isolated strains of virus from the various outbreaks The disease bears considerable resemblance clinically and epidemiologically to St. Louis encephalitis (Ch. XCIII) and the viruses have similar characteristics, although differing antigenically.

The best known outbreak of summer encephalitis occurred between July and September 1924 when some 7,000 persons, mainly in the Kagawa district, were affected (see Takaki, 1925, 1926 *a*, Kaneko, 1925). Since that date outbreaks of varying severity have occurred, but the incidence is variable and in some years only a few cases are reported. A useful general review of the disease is that of Warren (1946).

CLINICAL FEATURES

The characteristic clinical features have been recorded by a number of authors (e.g, Takaki, 1925, 1926 *a*, Kaneko, 1925; Kaneko and Aoki, 1928, who give an extensive Japanese bibliography, Naka, Kingo, and Kuroiwa, 1934, Hashimoto *et al*, 1936).

Onset. For a few days there may be prodromal symptoms of loss of appetite, drowsiness, and nausea, then comes the onset of the disease proper. The temperature rapidly rises and the patient becomes acutely ill Pains may be present in the head, chest, hips, or joints

The course is manifested by both encephalitic and meningitic symptoms

The encephalitic symptoms consist mainly of impairment of the mental faculties, at first there is restlessness, and even delirium, but after a few days apathy sets in. The patient's face is impassive and expressionless. In severe cases this apathy deepens into coma Various muscular movements such as athetosis, myoclonus, and twitchings of the face, hands, and tongue may occur On the other hand, aphasia, motor pareses of the extremities and tongue, and decreased response to painful stimuli may be found. The eye muscles are only rarely affected, apart from some slight contraction of the pupils

The meningitic reaction is evidenced by the presence of Kernig's sign, trismus, rigidity of the neck and limbs, and headache The abdominal reflexes are usually absent and the ankle and knee jerks exaggerated Incontinence or retention of urine may occur.

The fever usually lasts for 5 days or so to fall by lysis The course of the disease is seldom prolonged over 2 weeks If death occurs it is usually before the end of the second week

As regards residua, parkinsonism may occasionally be found, and there may be some persistence of headache, sleeplessness, irritability, neuralgia, neurasthenia, athetosis, pareses, and mental imbalance (see Sakurai and Ito, 1937)

Zimmerman (1946) reported the following features in a carefully studied outbreak on Okinawa: sudden onset with fever and headache, neck rigidity, Kernig's

sign, stupor, loss of speech, general convulsions, flaccid paralysis, Babinski's sign, and absent abdominal reflexes. In 4 proved cases on Korea, Sabin *et al.* (1947 *b*) referred to mental confusion, purposeless movements, convulsions, speech defects, deep stupor, or coma.

CLINICAL PATHOLOGY

Cerebrospinal fluid The cerebrospinal fluid is usually clear, the pressure moderately raised, and there is a pleocytosis up to 1500 cells per c.mm These cells are mainly lymphocytes, although in the early stage polymorphs may be found (Kaneko, 1925; Kaneko and Aoki, 1928, Hashimoto *et al*, 1936).

Blood The blood may show a polynucleosis up to 18,000 per c mm., although 11,000 is an average figure, the Schilling count shows a shift to the left (Matsumura *et al*, 1934, Hashimoto *et al.*, 1936). The red cells and hemoglobin may be slightly but correspondingly reduced, thus maintaining a color index of unity. In severe cases there is a moderate or pronounced acidosis with some hyperglycemia (Naka *et al*, 1934).

MORTALITY

The mortality has always been high, but to a certain extent varies from year to year Thus in 1873 the rate was 80 to 90 per cent., from 1901 to 1927, 55 to 60 per cent (Kaneko and Aoki, 1928), and in 1935 less than 30 per cent (Hashimoto *et al.*, 1936) These and many other authors have commented on the striking increase of severity with advancing age Thus, while under 11 the mortality rate was 47 per cent., from 11 to 20 years it was 40 per cent., and over 80 years it rose to 92 per cent.

PATHOLOGY

The pathological features have been recorded by a number of workers (e.g., Flexner, 1928, Kaneko and Aoki, 1928, Kingo, 1935 *a*, *b*, Hashimoto *et al*, 1936, Pette, 1938).

Naked-eye, the meninges are somewhat edematous, there is a moderate increase in the amount of cerebrospinal fluid, and congestion gives the gray matter of the cortex, basal nuclei, and pons a pinkish appearance. Histologically, perivascular cuffing with lymphocytes is found. There are also some focal infiltrations of mononuclears in the meninges, cortex, and brain stem. Degenerative changes are to be found in the nerve cells of the basal nuclei, pons, and medulla (particularly marked in the substantia nigra). Small foci of necrosis may be found. Bertrand and Miyashita (1936) have described cases in which definite plaques of demyelination were found.

Kingo, in particular, has described a diffuse polynuclear infiltration in the cerebrum, basal nuclei, and less commonly in the cord. He has also noted the occurrence of the iron reaction in the capillary endothelium and neighboring oligodendroglia

Zimmerman (1946) made a detailed study of some cases occurring on Okinawa Naked-eye, little was to be seen in acute cases, but in 3 persons living for more than a month, he found pale or "gritty" patches in the cortical and central gray matter, especially in the pallidal and red nuclei, thalami, and substantia nigra. Cystic changes were seen in the pallidal nuclei and substantia nigra. He described microscopic inflammatory and degenerative changes as recorded by the Japanese, and drew attention to neuronophagia of the anterior horn cells There was a widespread distribution of foci resembling those of disseminated sclerosis, limited to collections of ganglionic cells, in these areas there was an absence of myelin fibers In the cerebellum, there was injury to the Purkinje cells. The "gritty" foci were seen to be due to deposition of calcium, which led to a foreign body giant cell response

The features of a proved case in Korea were described by Sabin *et al* (1947 *b*).

Changes were limited to the gray matter and consisted of neuronal necrosis and neuronophagia. The lesions were present throughout the cerebral cortex, and in the Purkinje and molecular cell layers of the cerebellum.

As regards the relationship, on pathological grounds, between Japanese and von Economo's encephalitis, Kaneko and Aoki draw attention to the following comparatively unimportant points of difference: in Japanese encephalitis there is a greater tendency to formation of abscesses, a more widespread cellular infiltration, a more pronounced meningeal reaction, and more destruction of ganglion cells. If the reports of the occurrence of demyelination are confirmed then it would appear that there is a definite difference between the two histological pictures.

LABORATORY DIAGNOSIS

After death, the virus may be isolated from suspensions of brain and cord by inoculation in mice cerebrally, and in young mice peritoneally. Some mice will die after a few days. Brain suspension should be passed in series, and a portion used for neutralization and fixation tests with known sera (Sabin *et al.*, 1947 *b*).

In nonfatal cases, it may be worth while inoculating cerebrospinal fluid in a number of mice cerebrally and in young mice peritoneally.

The brain of fatal cases may perhaps contain complement fixing antigen, and it has been suggested that a direct fixation test be tried between this material and known serum (Sabin *et al.*, 1947 *b*).

As regards serological tests in nonfatal cases, the neutralizing antibody develops quickly and a very high neutralization index should be obtained after a comparatively short time. The complement fixation test is also of value.

EPIDEMIOLOGY

General Features

The disease has characteristically occurred in Japan in large epidemics. Practically every outbreak has taken place in the months of August and September, in the dry, hot weather. The most serious outbreaks occurred in 1924, 1927, 1929, and 1935 (see Iimura, 1936, Inada, 1938). Outbreaks have been rare of recent years. Thus, Sabin, Ginder, and Matumoto (1947) give these figures: 1939, 2,720, 1940, *529, 1941, 248, 1942, 407, 1943, nil, 1944, 12, 1945, 12.*

The highest incidence of cases is among peasants, but nonJapanese may be attacked.

As regards sex, there is a slightly increased liability to infection in men as *against women (1.26: 1, Kaneko and Aoki, 1928). Approximately 75 per cent* of cases have been reported as occurring in persons over the age of 40 (Naka, Kingo, and Kurowa, 1934), although there have been suggestions as to a recent alteration to involve children more frequently.

Geographical Incidence

Japan. The disease has been reported in many parts of Japan, especially around the Inland Sea, it occurs from Hokkaido in the North to the Ryukyu Islands in the South. Virus has also been isolated in Formosa (Kobayasi, 1940).

Korea. Hitherto this island has been thought to be free of the infection, but Sabin *et al.* (1947 *b*) described 4 cases in U.S. soldiers in 1946. Although native Koreans did not appear to suffer from encephalitis, a high percentage of adult sera neutralized the virus, suggesting a widespread dissemination of virus in the absence of epidemics—a position analogous to that present in China (see below).

Okinawa. Summer encephalitis is probably endemic in natives, and attacks the younger age groups, during World War II there were some military cases in U.S. forces, the evidence pointed to mosquito spread from horses or goats, some of

whose sera contained antibody (Hodes, Thomas, and Peck, 1945, 1946, Thomas and Peck, 1946, Zimmerman, 1946, Sabin, 1947 *b*).

China. Various reports confirm the presence of the infection in China, and the virus has been isolated and significant rises in serum antibody detected Thus encephalitis due to the Japanese virus has been identified as occurring in Peiping (Chu, Wu, and Teng, 1940, Huang and Liu, 1940, Huang, 1941, Yen, 1941), and Shanghai (Sabin, Schlesinger, and Ginder, 1947) It is probable that the virus is widely distributed in China, but that inapparent infections are more common than encephalitis Thus, Sabin, Schlesinger, and Ginder (1947) found virus neutralizing antibodies in the sera of 17/19 Chinese in Tientsin, and 11/13 in Shanghai. They quote Mitamura as having found that the sera of 83 per cent. of 104 normal persons in Nanking and Shanghai neutralized the virus

Russia autumn encephalitis A form of encephalitis occurring in the autumn in the Far East of Russia, and quite distinct from spring-summer encephalitis, is due to the virus of Japanese encephalitis The infection is spread by *Culex pipiens* and *C tritaeniorhynchus* (Smorodintsev, Shubladze, and Neustroev, 1940), other mosquitoes incriminated are *C. bitaeniorhynchus*, *Aedes japonicus*, and *Aedes esoensis* (Chagin and Kondratiev, 1943, Chagin, 1946).

Arthropod Transmission

[illegible] ed that the disease is *mosquito-* [illegible] (1937, 1938, 1939) has confirmed [illegible] (*Culex pipiens* var. *pallens*, and [illegible] outbreak transmitted the infection to mice by bite. (2) *Culex pipiens* var *pallens* mosquitoes were shown to become infected by feeding on virus suspension or by biting an infected mouse, they could then transfer the infection to fresh mice by biting (3) With *Culex pipiens* var *pallens*, they were able to demonstrate transovarial infection Russian observers have also incriminated mosquitoes in autumn encephalitis (see above)

In the United States, Reeves and Hammon (1946) undertook studies on mosquito transmission, with the possibility in mind that Japanese encephalitis might become established in North America They found that the following mosquitoes could be infected by feeding on virus suspension, and after a period of 6–25 days were infective for mice by biting *Culex pipiens var molestus, Culex pipiens* var. *pipiens, Culex quinquefasciatus, Culex tarsalis, Aedes nigromaculis, Aedes dorsalis, Culiseta incidens, and Culiseta inornata*

Bearing in mind the state of affairs in St Louis and equine encephalitis, it seems likely that there is some reservoir of infection in animals. The position is obscure, but antibodies have been detected in dogs (Mitamura *et al*, 1939), and in horses, goats, cattle, and rabbits (see above—Okinawa, also Sabin, Ginder, and Matumoto, 1947). Viremia occurs in experimentally infected horses (Thomas and Peck, 1946), chickens (Hammon, Reeves, and Burroughs, 1946), and virus persists in the blood of pigs and ducks for 24 hours after intravenous injection (Meiklejohn, Simpson, and Stacy, 1947)

It is obvious that much further work is required in Japan, analogous to that carried out by Hammon and his associates in America

It must not be overlooked that *ticks* may play some part Russian observers have found that virus may persist for several months in *H turkmeniense*, and transovarial infections may occur in this and other species (Chumakov, Petrova, and Sondak, 1945)

On the strength of finding antibodies in the sera of Japanese horses, goats, cattle, and rabbits, but not chickens or children, it has been suggested that in nonepidemic periods other arthropod vectors than mosquitoes may be involved, the possible rôle of helminths should not be overlooked in the transmission of infection between animals (Sabin, Ginder, and Matumoto, 1947).

PROPERTIES OF THE VIRUS

One of the earliest reports of the isolation of a virus from the 1924 cases was that of Kobayashi (1925). This virus aroused some interest at the time until Cowdry's (1927) discovery that it was a strain of rabies.

A large number of Japanese workers has isolated strains of virus from the brain or cerebrospinal fluid of cases since 1925, and the following is a list of many of these names· Takaki (1925, 1926 *a*, *b*); Ito *et al.* (1925 *a*, *b*), Nishibe (1925 *a*, *b*, 1926), Kojima and Ono (1925); Komatsu and Yamasaki (1925); Kaneko and Aoki (1928), Hayashi (1934), T...
Hashimoto *et al.* (1936); K...
hensive general review is gi...
of the strains isolated by th...
acteristics. These will now...

Animal Experiments

Rabbits Rabbits have been infected by a number of routes. e g, cerebral, corneal, intra-ocular, nasal, and testicular (Takaki, 1925, 1926 *a*, Nishibe, 1925 *a*, *b*, 1926, Taniguchi *et al*, 1935, Hashimoto *et al*, 1936, Kasahara *et al*, 1936 *b*, Kudo *et al.*, 1937) After peripheral injection, the virus can be found in the blood within 24 hours and remains there up to 4 days After intracerebral injection, the characteristic symptoms develop in a few days, namely, sleepiness, dyspnea, pupillary constriction, fever, and paresis of all limbs.

After intra-ocular injection, iridocyclitis occurs with glaucoma and atrophy. After corneal injection, keratoconjunctivitis develops. After testicular injection, orchitis develops in 3 to 5 days, but after repeated passage it occurs in 24 hours

Rabbits most frequently die in the 2nd week of the infection Histologically, after intracerebral injection, there is definite degeneration to be found in the cells of the midbrain and thalamus, there are also other characteristic encephalitic lesions The virus is found widely distributed in brain, cord, blood (both whole and plasma), and internal organs.

Guinea-pigs The virus can be passaged by intratesticular injection (Kasahara *et al*, 1936 *a*, *b*). Testicular virus can be transmitted to guinea-pigs by a number of other routes (Kasahara *et al*, 1936 *b*). Hammon and Espana (1947) report that only fever follows cerebral inoculation

Mice Encephalitis develops after intracerebral, nasal, intraperitoneal, testicular and subcutaneous injections (Kawamura *et al*, 1936 *a*, *b*, Kasahara *et al*, 1936 *a*, *b*, Taniguchi *et al*, 1936, Hashimoto *et al*, 1936, Kudo *et al*, 1937, Webster, 1938, Kobayashi, 1940, Lennette and Smith, 1940). After 3 to 4 days the fur becomes ruffled, the skin hyperesthetic, and tremors, convulsions, and pareses appear The animals frequently die, and microscopically the brain shows widespread perivascular ... tion The meninges
...idely distributed in
... virus passes to the
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of the central nervous system follows

As regards extraneural injection, it is chiefly young mice that are susceptible, resistance developing after about 2 weeks (Lennette and Koprowski, 1944)
... iturally infected with the virus
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Monkeys Monkeys can be infected by ocular and intracranial injection and by nasal instillation, young macaques being the most susceptible (Kaneko and Aoki,

1928, Hayashi, 1934, Taniguchi *et al*, 1935, 1936, Kawamura *et al.*, 1936 *a*, *b*; Webster, 1938, Kobayashi, 1940).

After corneal inoculation, Taniguchi *et al* discovered Guarneri-like bodies in the epithelium Up to 72 hours these bodies were intracellular, but thereafter they escaped and split up into elementary bodies of 0 25 μ diameter.

After intracranial injection, an incubation period of some 4 to 7 days elapses before symptoms appear, namely, neck rigidity, convulsions, and somnolence. Nystagmus, unequal pupils, ptosis, and salivation may be found Histologically, the pia is congested and infiltrated with lymphocytes Perivascular infiltration, nerve cell degeneration, neuronophagia and glial proliferation are widespread, but are most marked in the basal ganglia, midbrain, and pons. The virus is to be found in the blood and nasal washings of infected animals

After nasal inoculation the virus produces a local reaction, and then spreads to the brain via the olfactory nerves and bulbs Meningitis is found most marked basally, and infection of the whole brain follows thereafter.

Other animals The Syrian hamster is highly susceptible (Lennette, 1941). Sheep can be infected cerebrally or nasally (Webster, 1938). Chickens develop viremia after subcutaneous injection (Hammon, Reeves, and Burroughs, 1946). Transient viremia occurs in horses, pigs, and ducks after intravenous injection (see above). White rats can be infected by intratesticular injection (Kasahara *et al*, 1936 *b*)

Insusceptible animals The following usually prove refractory to injection of the virus dogs, cats, goats (Kawamura *et al*, 1936 *a*, *b*).

Morphology

Elementary bodies staining by Giemsa, and Guarnieri-like bodies in the epithelium were described in corneal preparations by Taniguchi *et al* (1935), but their significance is doubtful.

The virus passes the following filters Berkefeld N, W, Seitz EK, and Mandler (Takaki, 1926 *a*, Kawamura *et al*, 1936 *a*, *b*, Taniguchi *et al*, 1936, Kasahara *et al*, 1936 *a*, Kudo *et al*, 1937)

Filtration experiments show the size to be 20–30 mμ (Yaoi *et al*, 1939).

Cultivation

The virus has been grown in tissue culture (Haagen and Crodel, 1938, Kawakita, 1939).

Various workers have demonstrated the susceptibility of the fertile egg (Taniguchi *et al.*, 1936, Haagen and Crodel, 1938, Smith and Lennette, 1939) Of more recent years this question has been further explored, mainly with a view to vaccine production, and the following points have been demonstrated (Howitt, 1946, Koprowski and Cox, 1946 *a*, *b*, Morgan, Early, and McClain, 1946, Warren and Hough, 1946) The egg can be infected by all routes, especially by the yolk sac After inoculation, virus proliferates rapidly and reaches highest titer in the embryo Virus suspension can be titered, e g, by yolk sac inoculation Chick embryo tissue has been used in vaccine production (see below).

Reaction to Physical and Chemical Agents

The virus is destroyed at 55° C in ½ to 1 hour At 0° C. it survives for 3 weeks. It preserves its infectivity, especially in glass sealed tubes at −70° C. for a prolonged period, −20° C is not so efficient (Melnick, 1946) In glycerol it conserves its virulence for fully 7 months It is destroyed by bile (Takaki, 1925, 1926 *a*, Kudo *et al*, 1937) Virus loses its infectivity fairly quickly in saline, but proves more stable in 10 per cent serum or skimmed milk; the optimum pH is between 7 5 and 8 5 (Hirano and Koyama, 1939, Duffy and Stanley, 1945).

PROPERTIES OF THE VIRUS

One of the earliest reports of the isolation of a virus from the 1924 cases was that of Kobayashi (1925). This virus aroused some interest at the time until Cowdry's (1927) discovery that it was a strain of rabies.

A large number of Japanese workers has isolated strains of virus from the brain or cerebrospinal fluid of cases since 1925, and the following is a list of many of these names: Takaki (1925, 1926 *a*, *b*), Ito *et al.* (1925 *a*, *b*); Nishibe (1925 *a*, *b*, 1926); Kojima and Ono (1925); Komatsu [illegible] (1928); Hayashi (1934); Taniguchi *et al.* ([illegible] Hashimoto *et al.* (1936); Kasahara *et al* [illegible] hensive general review is given by Inada (1937). It may be said that the great bulk of the strains isolated by these workers resemble each other in their general characteristics These will now be described.

Animal Experiments

Rabbits. Rabbits have been infected by a number of routes: e.g, cerebral, corneal, intra-ocular, nasal, and testicular (Takaki, 1925, 1926 *a*, Nishibe, 1925 *a*, *b*, 1926, Taniguchi *et al.*, 1935; Hashimoto *et al*, 1936, Kasahara *et al.*, 1936 *b*, Kudo *et al.*, 1937). After peripheral injection, the virus can be found in the blood within 24 hours and remains there up to 4 days. After intracerebral injection, the characteristic symptoms develop in a few days, namely, sleepiness, dyspnea, pupillary constriction, fever, and paresis of all limbs

After intra-ocular injection, iridocyclitis occurs with glaucoma and atrophy. After corneal injection, keratoconjunctivitis develops. After testicular injection, orchitis develops in 3 to 5 days, but after repeated passage it occurs in 24 hours.

Rabbits most frequently die in the 2nd week of the infection. Histologically, after intracerebral injection, there is definite degeneration to be found in the cells of the midbrain and thalamus, there are also other characteristic encephalitic lesions The virus is found widely distributed in brain, cord, blood (both whole and plasma), and internal organs.

Guinea-pigs. The virus can be passaged by intratesticular injection (Kasahara *et al*, 1936 *a*, *b*). Testicular virus can be transmitted to guinea-pigs by a number of other routes (Kasahara *et al*, 1936 *b*) Hammon and Espana (1947) report that only fever follows cerebral inoculation.

Mice. Encephalitis develops after intracerebral, nasal, intraperitoneal, testicular and subcutaneous injections (Kawamura *et al*, 1936 *a*, *b*, Kasahara *et al.*, 1936 *a*, *b*, Taniguchi *et al*, 1936, Hashimoto *et al*, 1936, Kudo *et al.*, 1937, Webster, 1938, Kobayashi, 1940, Lennette and Smith, 1940) After 3 to 4 days the fur becomes ruffled, the skin hyperesthetic, and tremors, convulsions, and pareses appear. The animals frequently die, and microscopically the brain shows widespread perivascular lymphocytic cuffing, lymphocytic infiltration, and glial proliferation. The meninges are hyperemic and infiltrated with lymphocytes The agent is widely distributed in the brain, blood, and internal organs After nasal instillation, the virus passes to the base of the brain by the olfactory nerves and bulbs, and a generalized infection of the central nervous system follows.

As regards extraneural injection, it is chiefly young mice that are susceptible, resistance developing after about 2 weeks (Lennette and Koprowski, 1944) [illegible] iturally infected with the virus [illegible] on various experiments using [illegible]

[illegible] l intracranial injection and by nasal instillation, young macaques being the most susceptible (Kaneko and Aoki,

on the 5th day of the disease, indicating that antibody can play little part in protection.

Antibody also develops after subclinical attack (Kudo *et al.*, 1937, Mitamura *et al*, 1939). Recent observations in Japan suggest that antibody is now very rare in children under 10, but is present in many adults (Sabin, Ginder, and Matumoto, 1947)

These antibodies are tested by the cerebral route in adult mice (Olitsky and Casals, 1947, see Ch XI; Sabin, 1947 *b*) or by inoculation subcutaneously in 3-day mice (Lennette and Koprowski, 1946), or peritoneally in somewhat older mice.

Certain observations suggest that normal animal and human serum may contain nonspecific virus neutralizing substances (Kasahara *et al*, 1937 *a*, Sabin, 1943) Koprowski (1946) found that the sera of certain marsupials and rodents captured in Brazil, chiefly *Didelphis marsupialis*, and members of the genus *Oryzomys*, that neutralized the virus of yellow fever, also inactivated one or more of Japanese B, St. Louis, and West Nile viruses, not known to occur in Brazil This nonspecific substance was not destroyed at 56° C A lipoid fraction of normal animal serum rapidly inactivates Japanese virus (Casals and Olitsky, 1947).

It has been shown with rabbit sera that the gamma globulin fraction is protective and not the albumin (Koprowski, Richmond, and Moore, 1947).

Complement fixation occurs between [illegible] itable antigens Thus [illegible] More lately various [illegible] freezing and thaw- [illegible] exposure to UVL (Havens *et al*, 1943), by heating and clarification by centrifugation (Casals, 1945), and by a mixture of the above methods, the resultant antigen being lyophilized for storage (Casals, 1947, see also Ch XI) DeBoer and Cox (1947) claim to have produced a highly specific antigen by extraction in the lyophilized state with benzene, toluene, or dichlorethylene This procedure has been modified by Espana and H[illegible] 47) They prepare high titer [illegible] r develop- ing (Hammon [illegible] in man a few days after [illegible] some weeks (Zimmerman, 1946, [illegible] Simpson, and Stacy, 1947; Sabin, 1947 *b*, Sabin *et al*, 1947 *b*) Sabin points out that the virus neutralizing antibody persists longer than the complement fixing, and that the presence of high titer complement fixing antibodies suggests a recent infection (see also Sabin *et al*, 1947 *b*).

Precipitation has been reported between serum and a filtered boiled brain suspension (Takaki, 1925)

RELATIONSHIP TO ST. LOUIS ENCEPHALITIS AND ENCEPHALITIS LETHARGICA

On clinical and epidemiological grounds, Japanese Type B encephalitis closely resembles St Louis encephalitis Pathologically it presents points of resemblance both to this disease as well as lethargic encephalitis

The Japanese virus resembles very [illegible]

[illegible] does not protect against injec- [illegible] virus, although Japanese antiserum (animal) may show some protective properties against both viruses Of recent years it has been found that the West Nile virus is also related, and it appears reasonable to place the 3 in a subgroup, Japanese virus being the most comprehensive antigenically, West Nile being the least comprehensive, with St Louis occupying an intermediate position (see Ch XLVII for a full discussion).

It may be concluded, therefore, that the two forms of encephalitis, Japanese and

IMMUNITY

Active Immunity

Animals that recover from experimental infection become immune to reinoculation. This result can also be brought about by the administration of a vaccine, or of live virus by a route that does not normally lead to a fatal outcome In man, immunity almost certainly arises in many instances by subclinical infection During World War II, American investigators explored the possibility of producing vaccines on a large scale, for use in Japan and other endemic areas in the Far East. Two vaccines were produced.

Mouse brain vaccine. Experiments of Duffy and Stanley (1945) suggested that it was not possible to secure by the centrifugation of mouse brain a preparation of virus sufficiently concentrated and free from mouse tissue to be used for vaccine production on a large scale. However, Sabin and his associates have introduced a relatively crude form of vaccine that has been used on a fairly large scale by American forces (Sabin, 1943, 1947 *b*). The vaccine consists of 10 per cent mouse brain suspension inactivated with 0 2 per cent. formaldehyde. Injected in mice in 2 doses, the vaccine stimulated resistance to infection by the peritoneal route, and injected in larger doses, immunized against cerebral or nasal challenge.

Two or 3 injections are given in man. There have been no reports of demyelinating reaction (Sabin, 1947 *b*). About 50 per cent. of persons vaccinated develop neutralizing antibodies after the 3rd dose, complement fixing antibodies rarely develop (Ginder *et al.*, 1947, Sabin, 1947 *a*, Sabin and Duffy, 1947). Sabin *et al* [illegible] 's compared to persons [illegible] not the older people, [illegible] who had neutralizing antibody prior to inoculation (presumably due to old infection) but not in old people without such antibody.

Chick embryo vaccine Warren and Hough (1946) worked with homogenized infected embryos, and added formalin to a final concentration of 0 2 per cent., the final concentration of chick tissue being 10–20 per cent The vaccine was shown to be antigenic in mice A similar type of vaccine was used by Koprowski and Cox (1946 *b*) Using the method of assay introduced by Sabin (above), the chick vaccine did not reach the specified requirements However, mice vaccinated with chick vaccine showed a much higher degree of resistance when challenged with live homologous (chick cultivated) virus, and not with mouse brain If mice were vaccinated peritoneally with chick vaccine, and challenged intravenously with chick cultivated virus, the immunogenic powers of chick and mouse vaccines were seen to be comparable The vaccine may be given subcutaneously in 2 doses at a week's interval, with a 3rd dose after 4 weeks

Serum Antibodies

Virucidal antibodies develop in the serum of convalescent animals, and after vaccination (Takaki, 1925, 1926 *a*, *b*, Kawamura *et al*, 1936 *a*, *b*, Hashimoto *et al*, 1936, Kudo *et al*, 1937, Kasahara *et al*, 1937 *a*, *b*, Haagen and Crodel, 1938, Smith and Reames, 1940). Antibodies, presumably due to specific infection, have been found in the sera of horses, goats, cattle, and rabbits, but not chickens, in endemic areas (Sabin, Ginder, and Matumoto, 1947)

In man, the antibody usually appears about 7 days after onset of encephalitis, but may be delayed for some weeks, it persists for at least 5 years, perhaps for life (Takaki, 1926 *a*, Kawamura *et al*, 1936 *a*, *b*, Taniguchi *et al*, 1936, Kudo *et al*, 1937, Sabin, 1947 *b*).

Sabin *et al.* (1947 *b*) describe a fatal case showing very high neutralizing indices

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St. Louis, bear close resemblances to one another and differ considerably from encephalitis lethargica.

RELATIONSHIP TO OTHER VIRUSES

Japanese encephalitis antisera do not neutralize the following neurotropic viruses, Levaditi's "encephalitis" virus, herpes febrilis, Koritschoner's strain, *virus fixe*, and neurovaccine (Kudo *et al*, 1937). The virus is not related to Anopheles A virus (Roca-Garciá, 1944), Bunyamwera virus (Smithburn, Haddow, and Mahaffy, 1946), or Colorado tick fever virus (Koprowski and Cox, 1947). The finding by Webster (1938), that sheep are susceptible, introduces the question of relationship to louping ill, and therefore to the related virus of Russian encephalitis. This question is fully discussed in Ch. XCVIII, where it is shown that although Russian observers claim that Russian and louping ill viruses are related to the Japanese subgroup, Casals does not agree.

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CHAPTER XCV

THE EQUINE ENCEPHALOMYELITIS GROUP OF VIRUSES. INTRODUCTORY

THERE is a number of viruses that cause encephalomyelitis in horses and mules In Europe, the infection is usually known as Borna disease, due to a serologically distinctive virus (see Howitt and Meyer, 1934)[1]; this virus is not thought to attack man, and will not be further discussed.

Another variety of equine encephalitis occurs in Russia, this virus is also antigenically distinctive (Howitt, 1935, 1937 *b*).

The equine encephalomyelitis viruses with which we shall be concerned all occur in the Western Hemisphere, and are as follows· North American equine encephalomy[illegible] types, the eastern and [illegible] virus, and the Venezue[illegible] and is described in Chapter XCVII

We shall now discuss the interrelationships of the eastern and western types of virus, the Brazilian and the Argentine viruses

Antigenic Relationship between Eastern and Western Types

The biological properties of the two North American types are closely similar, but the eastern type induces a more severe disease in horses and men

Numerous observations have been made on the antigenic relationships of the 2 strains, and it appears quite justifiable to refer to them as distinct eastern and western types of one North American virus Sharing of group antigens between the two types is best brought out by the complement fixation test (Howitt, 1938 *b*, Havens *et al*, 1943)

Cross reactions can be shown under certain conditions by the virus neutralization technique, especially if hyperimmune sera are employed (Howitt, 1938 *b*).

In general, the most satisfactory technique for "typing" strains, to elicit the maximum difference in antigenic composition, is to make use of cross resistance tests Such tests are frequently performed in guinea-pigs, and these animals when immunized to one type are usually fully susceptible to the other (see, e g, Records and Vawter, 1935), even so, some cross resistance may be demonstrable (Howitt, 1935) In immunized horses, the types prove specific, and can be differentiated, but on occasion cross resistance may be demonstrable (Records and Vawter, 1934 *b*, 1935, Shahan and Giltner, 1935 *a*, Shahan *et al*, 1942). Mice may be used in typing tests, and specificity has been demonstrated by the use of unweaned baby animals (Wright, 1942, Lennette and Koprowski, 1945)

Antigenic Structure of Strains of North American Virus

Although no considerable series of tests has been reported, observations do not suggest that there is any antigenic heterogeneity in western or in eastern strains (Ten Broeck and Merrill, 1933, Howitt, 1935, Beck and Wyckoff, 1938, Olitsky, Morgan, and Schlesinger, 1945).

The Brazilian Strain of Virus

A virus isolated from equine encephalomyelitis in Brazil has been found to be a strain closely resembling, but not identical with, the eastern type of North American virus (Carneiro, 1937, Carneiro and Cunha, 1943).

[1] References are appended at the conclusion of Ch XCVI, p 1132 et seq

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CHAPTER XCVI

NORTH AMERICAN EQUINE ENCEPHALOMYELITIS: EASTERN AND WESTERN TYPES

During the last few years, equine encephalomyelitis has developed into the major problem of the horse industry in North America, and there have been hundreds of thousands of equine deaths.

There are two antigenically distinct varieties of the North American equine encephalomyelitis virus. The western type was first isolated by Meyer, Haring, and Howitt (1931) and the eastern by Ten Broeck and Merrill (1933) and Giltner and Shahan (1933 *a*).

SOME FEATURES OF THE DISEASE IN EQUINES

Incidence

The infection (western type) was prevalent in California in 1930–31 (Meyer, 1933 *b*) In 1933 the eastern type of virus was prevalent in Virginia, Delaware, and Maryland (Giltner and Shahan, 1933 *a, c*).

Miller (1945) gives the following figures for the number of cases since 1935

1935	23,512	1940	16,941
1936	3,929	1941	36,872
1937	173,889	1942	4,939
1938	184,662	1943	4,768
1939	8,008	1944	19,590

In 1945 there were 3,212 cases (Simms, 1946)

The marked reduction in 1939 is ascribed partly to an increased resistance induced by earlier attacks, but more especially to the introduction and vigorous adoption of a policy of vaccination, using chiefly formolized chick embryo vaccine (*Public Health Reports*, 1940, 55, 601).

Fatality

The fatality rate for infection due to the eastern virus is about 90 per cent, and for that due to the western virus about 27 per cent (see, e g, Giltner and Shahan, 1933 *a*, *Public Health Reports*, 1940, 55, 601).

Geographical Distribution

The United States.

Until 1939, the Appalachian Mountains served as a barrier between the area on the Pacific side where infection was due to the western type, and the Atlantic area where the eastern type was found However, in the last few years, there has been a westward spread of the eastern type.

The western type of virus has been identified in the following states as the cause of equine epizootics (Mohler, 1943).

The Argentine Strain of Virus

Equine encephalomyelitis occurs in the Argentine, and, strangely, the virus has been found to be closely related to the western North American type, it may be regarded as a strain of the western type (Meyer *et al*, 1934; Rosenbusch, 1934, Howitt, 1935). This virus has biological properties characteristic of the equine encephalomyelitis group, and, for example, infects many animals such as rabbits, guinea-pigs, dogs, cats, rats, mice, goats, hedgehogs, pigeons, storks, geese, ducks, hawks and blackbirds, the cranial route is usually used, but in some cases a peripheral route secures infection (Rosenbusch, 1934, Remlinger and Bailly, 1935 *a, b*, 1936 *a, b, d*). Other properties are also characteristic of the group (Remlinger and Bailly, 1936 *c*).

We shall now proceed to a description of the North American equine encephalomyelitis, and after that to Venezuelan equine encephalomyelitis.

Eastern Type

Cases of human infection due to the eastern type of virus were first repor from Massachusetts and New England in 1938 (Feemster, 1938, Fothergill et 1938, Webster and Wright, 1938, Wesselhoeft, Smith, and Branch, 1938, McAd and Porter, 1939, Farber *et al*, 1940, Getting, 1941). Further cases have occur since that time in the country east of the Appalachian Mountains (see, e g, B and Irons, 1942, Hammon, 1943). In the 1938 outbreak, the disease was freq in children, and ran a severe course (Farber *et al*, 1940, Getting, 1941). T showed fever, vomited, and became drowsy. Shortly, localized twitchings, aph diplopia, paralyses, and general tonic or clonic convulsions occurred Nerv sequelae were recorded

DIFFERENTIAL DIAGNOSIS

Clinically it is difficult to distinguish between equine encephalitis and infect due to the St. Louis virus, as has been emphasized by various authors (Buss Howitt, 1941, Hammon, 1943; Eklund, 1946) Epidemics may occur in the s area and at the same time. In fact, double infections may occur in the one pati

Abortive poliomyelitis presents clinical features similar to those of mild subcl cal encephalitis, and confusion may arise in the absence of adequate laborat tests (Buss and Howitt, 1941; Adamson and Dubo, 1942 *a*, *b*, Jackson, 1942). Minnesota, Eklund (1946) drew attention to the coexistence of poliomyelitis encephalitis Useful distinctions were that 67 per cent of poliomyelitis cases curred after August 23rd, but only 6 per cent of encephalitis cases. Poliomye occurred mostly under 20 years of age

LABORATORY INFECTIONS

A few laboratory infections due to the western virus have been repor (Fothergill, Holden, and Wyckoff, 1939, Helwig, 1940).

In these cases it is probable that the portal of entry has been the respirat tract.

DISTRIBUTION OF THE VIRUS IN MAN

Virus can be recovered from the brain and cord of fatal cases The west virus has been found in the CSF (Fothergill, Holden, and Wyckoff, 1939, Ch and Norris, 1942, Gwatkin and Moynihan, 1943) As regards the blood, Ho (1939 *a*) recovered the western type from serum

CLINICAL PATHOLOGY

The CSF in the western infection shows pleocytosis, up to some hundreds cells per c mm Polymorphs are found in the first few days, but are later repla by

and

and

show a moderate polymorph leukocytosis

PATHOLOGY

Eastern Type

The pathology has been described by Fothergill *et al.* (1938), Eklund Blumstein (1938), and Farber *et al* (1940), but chiefly by Wesselhoeft, Smith, Branch (1938) The following account is mainly that of the last-mentioned auth

Brain With the naked eye, the meningeal vessels were seen to be congest especially overlying the brain stem, on section the brain was pinkish and s

Alabama	Iowa	Nevada
Arizona	Kansas	North Dakota
California	Kentucky	South Dakota
Colorado	Michigan	Texas
Idaho	Minnesota	Utah
Illinois	*Montana*	*Washington*

The eastern type of virus has been identified in the following states as the cause of equine epizootics (Mohler, 1943):

Alabama	Georgia	N Carolina
Connecticut	Massachusetts	S. Carolina
Delaware	Michigan	Texas
Florida	New Jersey	Virginia

In addition, Miller (1945) records the isolation of this type in Missouri, and it has been found in Panama (Kelser, 1937 *b*).

It will be observed that in the States of Alabama, Michigan, and Texas both types of virus have been described (see also, Randall and Eichhorn, 1941, Shahan and Giltner, 1943, Brown, 1947 *b*).

Canada.

Despite the proximity to America, the infection has not presented such a problem, although outbreaks of the disease, some serious, have been described from time to time, e.g, *Western type:* 1936, 1937, Saskatchewan (Fulton, 1938), 1938, Saskatchewan, where over 15,000 animals died (Fulton, 1941, Gareau, 1941), Ontario (Schofield and Potter, 1938), 1941, Manitoba (Bowman, 1945, see also Savage, 1942) *Eastern type.* 1938, Ontario (Schofield and Labzoffsky, 1938).

NORTH AMERICAN EQUINE ENCEPHALOMYELITIS IN MAN: CLINICAL FEATURES

Western Type

Meyer (1932, 1933 *a*) described the first cases in man. In California, many more cases have been reported since 1937 (Howitt, 1938 *a*, 1939 *a*, *b*, 1942, Buss and Howitt, 1941) In 1937 and 1938 a few cases occurred in Minnesota (Eklund and Blumstein, 1938), 800 cases were reported in 1941 (Eklund, 1946) Other outbreaks have occurred as follows Arizona (Meiklejohn and Hammon, 1942), N Dakota (Breslich, Rowe, and Lehman, 1939), Texas (Wheeler, 1941, Hammon, 1943, Sulkin, 1946) Leake (1941) referred to outbreaks in N and S Dakota, Minnesota, Manitoba, Montana, and Nebraska There have been many cases reported from the Canadian Prairie Provinces, especially in 1941 (Fulton, 1941, Gareau, 1941, Adamson and Dubo, 1942 *a*, *b*, Cameron, 1942, Davison, 1942, Donovan and Bowman, 1942 *a*, *b*, Jackson, 1942, 1943, McGugan, 1942, Norris, 1946) There was an *outbreak in 1947 in Manitoba (Bowman, 1947)*

The onset is sudden, with headache, sweating, disturbance of sleep, confusion, and drowsiness Pain and stiffness in the neck and back may occur There may be nystagmus, tremors, and paralyses The illness lasts for a week or so Spastic paralysis has been recorded as a sequel, especially in infants Davis (1940 *a*) described cerebral atrophy, mental deterioration, blindness, deafness, speech defect, and partial hemiplegia. Nineteen cases in infants were described by Medovy (1942), 6 showing sequelae Later (1943) he described further cases. Sequlae have also been described by Platou (1940) The mortality is about 10 per cent.

Mild subclinical cases of encephalitis resemble abortive poliomyelitis (Adamson and Dubo, 1942 *a*, *b*).

districts (Fulton, 1941; Richter, 1942), or from desert or mountainous country (Buss and Howitt, 1941). There is not usually any close connection between sick horses and human cases Familial cases are rarely encountered. The disease occurs from May to September, chiefly in July and August. For example, in the 1941 Minnesota outbreak, 91 3 per cent. of cases had onset between July 6 and August 23 (Eklund, 1946).

As regards the age and sex incidence, in certain outbreaks there has been a marked tendency for cases to occur predominantly in adult males Thus in 1941 in N. Dakota and Minnesota, about 70 per cent. of cases were males (Leake, 1941, Eklund, 1946) Infants seem also to be frequently attacked (Buss and Howitt, 1941; Jackson, 1943). Hammon (1943) concludes that age and sex incidence varies in different areas, regardless of which virus is concerned He points out that environmental factors of exposure strongly influence age and sex incidence.

The Rôle of Mosquitoes

The seasonal incidence of the disease suggests that an insect vector is concerned, and, following numerous investigations, this suggestion has been abundantly confirmed

The methods used in the study of the part played by mosquitoes include isolation of virus from pools of trapped mosquitoes, and transmission of the disease from animal to animal by artificially infected mosquitoes

In assessing the importance of any one species, one must pay attention to its abundance, feeding habits (which can be investigated by precipitin tests on blood meals), range of flight, and breeding place.

It is particularly important to know the feeding habits of mosquitoes, for example *Aedes* prefer mammalian to avian blood, whereas *Culex* feed on birds and mammals (see Bang and Reeves, 1942, *California Mosquito Control Association*, 1942). However, Aedes may sometimes feed on chickens (Hammon and Reeves, 1947).

The technical details of mosquito transmission experiments, and of the isolation of virus from pools of mosquitoes have been described (Knowlton and Rowe, 1935, Kelser, 1940*a*, Hammon, Reeves, and Izumi, 1942, Reeves and Hammon, 1942)

Recovery of Virus in Nature

Virus has been recovered from pools of trapped mosquitoes in endemic areas, for example from *Aedes dorsalis*, western virus (Hammon, Reeves, and Galindo, 1945*a*, and see San Joaquin Valley), *Anopheles maculipennis freeborni*, western virus (Hammon *et al*, 1945), *Culex pipiens*, western virus (Hammon *et al*, 1945), *Culex restuans*, western virus (Norris, 1946), *Culex stigmatosoma*, western virus (see San Joaquin Valley), *Culex tarsalis*, western virus (Hammon, Reeves, and Galindo, 1945*b*, Hammon and Reeves, 1947, McLintock, 1947, and see Yakima Valley and San Joaquin Valley), *Culiseta inornata*, western virus (Hammon *et al*, 1945)

Virus has been recovered from *Culex tarsalis* on no less than 75 occasions (see Hammon and Reeves, 1945) Hammon and his associates have tested over 25,000 *Aedes mosquitoes*, but have isolated the western virus only twice, i.e., 1 12,500, (see Hammon and Reeves, 1945) In contrast, the rate for all *Culex tarsalis* tested during a 3-year period was 1 225 It is of interest to note that a high infection rate of *C tarsalis* is not necessarily concurrent with a high attack rate in man (Hammon and Reeves, 1947)

Experimental Transmission Experiments

Various mosquitoes have been shown experimentally to be capable of transferring infection from an infected animal or virus suspension to a healthy animal by bite; guinea-pigs and fowls have been much used in this type of work. The

Microscopically, the subarachnoid space was infiltrated with lymphocytes and some mononuclears and polymorphs, embedded in a fibrinous coagulum.

Perivascular cuffing with lymphocytes, polymorphs, or mononuclears was a prominent feature, some plasma cells also occurring, there were some perivascular hemorrhages. *Polymorphs often infiltrated the walls of the vessels, and filled the* perivascular spaces.

Diffuse infiltrations were found most markedly in the severer cases, in the cortex, basal ganglia, pons, and down to the level of the cervical cord. The infiltrating cells were mainly polymorphs, but some lymphocytes and mononuclears also occurred.

Nerve cell degeneration was a characteristic feature, and all stages up to slight neuronophagia might be seen. Lesions were seen in the medial lemniscus, transverse fibers, and the nuclei of the pons and cranial nerves, rarely was there degeneration of the Purkinje cells.

Cord. Lesions in the cord were less pronounced than in the brain. Meningeal and diffuse cellular infiltrations were found; there was also nerve cell degeneration, especially in the cervical cord.

The changes were those of an acute disseminated encephalomyelitis pronounced in the basal ganglia, pons, and medulla. The reactions were more severe than occur in Japanese or St. Louis encephalitis, and did not resemble encephalitis lethargica or postvaccinal encephalitis

Other organs. Fothergill *et al.* (1938) reported the presence of an early interstitial pneumonitis Wesselhoeft, Smith, and Branch found general hyperemia of the abdominal and thoracic viscera, anasarca, and toxic appearances in the kidneys, spleen, and liver, there was also enlargement of the lymph glands.

Western Type

The pathological features have been described by Baker and Noran (1942), Peers (1942), Quong (1942), and Noran (1944). The meninges were found to be edematous and showed a mild mononuclear infiltration. Perivascular cuffing was with round and microglial cells, although polymorphs might occur The lesions in the brain and cord were characteristically small and focal Petechial hemorrhages and areas of demyelination were seen in some cases Peers refers to the fact that the majority of infiltrating cells is mononuclear, in contrast to the predominance of polymorphs described in eastern infections Noran refers to chronic changes such as cystic degeneration of the left frontal and temporal lobes, parenchymal degeneration, and focal areas of inflammatory infiltration. Many vessels were occluded by endothelial proliferation and calcium deposits.

LABORATORY DIAGNOSIS

In fatal cases, brain and cord suspension is inoculated cerebrally in mice and guinea-pigs To "type" the strain, inoculation is made in guinea-pigs immunized against the western or eastern type separately In other cases, inoculation of CSF is made, but the virus is not uniformly present The inoculation of the chick embryo has been specially recommended (Fulton, 1941)

Main reliance is placed on the virus neutralization or complement fixation test carried out with acute and convalescent phase sera (Jakmauh and Feemster, 1939, Buss and Howitt, 1941, Adamson and Dubo, 1942 *a*, *b*, Casals, 1941, 1945 *b*, Gold and Hampil, 1942, see also Ch XI)

EPIDEMIOLOGY

Incidence

Epidemic equine encephalomyelitis in man is predominantly a disease of the countryside, especially farming areas It has only rarely been reported from urban

infection is transmitted between these animals largely by mosquitoes, and to a lesser degree by mites and ticks

The evidence supporting the rôle of animal reservoirs is as follows

The eastern virus has been recovered from pheasants (Tyzzer, Sellards, and Bennett, 1938, Van Roekel and Clarke, 1939, Beaudette, Black, and Hudson, 1941, Sellards, Tyzzer, and Bennett, 1941); and from pigeons (Fothergill and Dingle, 1938). The sera of various wild fowl have been found to neutralize the virus (Brown, 1947 *b*), and it has been shown that sparrows and pigeons can be infected by the bite of an infected mosquito, infected birds can serve as a source of infection for mosquitoes (Davis, 1940 *b*) As regards the western virus, the observations of Hammon and his associates, mainly on the Pacific coast of America, chiefly by means of serum tests, have pointed to infection of mammals and birds (especially fowls) with this and the St. Louis virus (Hammon *et al.*, 1941 *a*, Howitt and van Herick, 1941, 1942; Hammon and Reeves, 1943, 1947) Hammon and Reeves (1946) showed that viremia was readily produced in chickens inoculated subcutaneously. The virus may be spread between chickens by mosquitoes, especially *Culex tarsalis*, and these infected mosquitoes may also bite horses and men The virus may also be spread from chicken to chicken by the chicken mite *Dermanyssus gallinae* (Sulkin, 1945).

The western virus has also been recovered from the naturally infected prairie chicken *Tympanuchus cupido americanus* Reichenbach (Cox, Jellison, and Hughes, 1941), and from a pig (McNutt and Packer, 1943) Some evidence has been produced that gophers (*Citellus richardsoni*) may be infected (Gwatkin and Moore, 1940, Gwatkin and Moynihan, 1942). Serological tests do not suggest that wild rodents and rabbits play much part, but antibodies to the California virus are not uncommon (Hammon and Reeves, 1947).

It is evident, therefore, that the virus of equine encephalitis is widely distributed in nature. Infected animals show a short period of viremia, and during this phase mosquitoes may become infected.

Owing to the probable short duration of the viremia in animals, it would appear that the main "reservoir" of the virus must be in mosquitoes, and that the virus may survive in them from one season to the next It is possible that ticks or mites serve as reservoirs of infection for animals, especially fowls Although in certain areas the evidence incriminating the fowl is strong, in other districts, for example in Nebraska and Texas, there is less certainty, and other hosts may be concerned

MOSQUITO TRANSMISSION IN CERTAIN LOCALITIES IN NORTH AMERICA

A. Western Districts

1 Yakima Valley.

Hammon and his associates have made detailed studies in this locality in the State of Washington, where there has been a number of outbreaks of human encephalitis due to the St Louis virus and to the western virus, and in some cases there has been a double infection (Hammon, 1941, 1943, Hammon and Howitt, 1942, Hammon *et al.*, 1945) Encephalitis has also been noted in equines

The human cases have occurred mainly in or near orchards The onset has been in July, reaching a peak in August, and then rapidly declining

Hammon and Reeves (1947) give the following as the number of clinically recognized human cases of encephalitis, 1938, 4, 1939, 31, 1940, 58, 1941, 26, 1942, 28, 1943, 1, and 1944, 0.

The conditions in this hot irrigated valley are specially favorable for mosquitoes, and the seasonal incidence pointed to their part in the spread In support, western virus (and St Louis virus) has been isolated from pools of *Culex tarsalis* mosquitoes

following have been shown capable of transferring the infection in this way: *Aedes aegypti*, eastern and western virus (Kelser, 1933, 1935; Merrill and Ten Broeck, 1934, 1935; Davis, 1940 *b*); *Aedes albopictus*, western virus (Simmons, Reynolds, and Cornell, 1936), *Aedes atropalpus*, eastern virus (Davis, 1940 *b*); *Aedes cantator*, eastern virus (Merrill, Lacaillade, and Ten Broeck, 1934); *Aedes dorsalis*, western virus (Herms, 1933, Madsen and Knowlton, 1935), *Aedes lateralis*, western virus (Reeves, 1941); *Aedes nigromaculis*, western virus (Madsen and Knowlton, 1935), *Aedes sollicitans*, both types (Merrill, Lacaillade, and Ten Broeck, 1934); *Aedes sylvestris*, western virus (Merrill, 1933), *Aedes taeniorhynchus*, western virus (Merrill, 1933, Kelser, 1937 *a*, 1938); *Aedes triseriatus*, eastern virus (Davis, 1940 *b*), *Aedes vexans*, eastern and western virus (Knowlton and Rowe, 1935; Davis, 1940 *b*); *Culex tarsalis*, western virus (Hammon and Reeves, 1943; Hammon, Reeves, and Gray, 1943); *Culiseta incidens*, western virus (Hammon and Reeves, 1943), *Culiseta inornata*, western virus (Hammon and Reeves, 1943).

Hammon and Reeves (1945) quote Japanese workers as having reported transmission of the western virus with *Culex pipiens* var. *pallens*, and *Armigeres obturbans*. The same workers claim to have transmitted the eastern virus by *Culex pipiens* var *pallens*, and *Culex tritaeniorhynchus*

A number of workers has adduced evidence that encephalomyelitis viruses proliferate in the tissues of the following mosquitoes, and increase in amount up to ten thousand fold *A sollicitans*, *A. aegypti* (Merrill, Lacaillade, and Ten Broeck, 1934, Merrill and Ten Broeck, 1934).

The virus appears to be well distributed through the tissues of the infected mosquito

It has been found that the western virus increases in titer when cultured in the presence of mosquito tissues (Trager, 1938)

The mosquito probably becomes infective about 7–20 days after the infecting meal, at a suitable temperature, and remains so for the rest of its life, transovarial infection apparently does not occur (Merrill, Lacaillade, and Ten Broeck, 1934; Merrill and Ten Broeck, 1935)

Probably because the titer of virus in the blood of the horse is so low, and the period of viremia short, no one has succeeded in infecting a mosquito by allowing it to bite a horse

THE RÔLE OF OTHER ARTHROPODS

Ticks have been incriminated Thus, the western virus can be passed hereditarily in *Dermacentor andersoni* Stiles (Syverton and Berry, 1941 *b*), larvae nymphs, and adults can all infect by feeding These ticks became infected after feeding on a gopher (Gwatkin, 1939).

The western virus has been isolated from *Triatoma sanguisuga* Le Conte gathered in Kansas, this tick feeds largely on vertebrates (Kitselman and Grundmann, 1940). It can transmit virus experimentally in guinea-pigs (Grundmann *et al*, 1943) Experiments of these workers did not suggest that *D. variabilis* Say (the dog tick) is of importance as a transmitter of infection

Mites have also been shown capable of transmitting the infection Thus western virus has been recovered from mites *Liponyssus sylviarum* from the nest of a blackbird in Kern County, California, from the nest of an English sparrow, virus was recovered from this mite and from *Dermanyssus americanus* Ewing (Reeves *et al*, 1947). The virus has also been recovered from the chicken mite *Dermanyssus gallinae* (Sulkin, 1945), but studies in Yakima have proved negative (Hammon and Reeves, 1947)

Animal Reservoirs of Infection

There is definite evidence that the virus of equine encephalomyelitis infects not only equines and man, but many domestic and wild animals It is probable that

species They drew attention to the influence of altitude on the distribution of the disease by its effect on limiting the flight of the probable vector *C. tarsalis*

B. Eastern Districts

1. Massachusetts.

In the 1938 outbreak, it was concluded that *Aedes vexans* was the most likely vector, *A. sollicitans*, and *A. cantator* may have been partly responsible (Getting, 1941; Feemster and Getting, 1941). Davis (1940 *b*) found that the following mosquitoes native to Massachusetts could transmit the eastern virus from infected animals to others experimentally. *A. vexans, A. sollicitans, A cantator, A. atropalpus,* and *A triseriatus*. In addition, a laboratory-reared *A. aegypti* could transmit the infection. *Culex, Mansonia,* and *Anopheles* gave negative results. He also concluded that *A vexans* was the main vector.

2. Oklahoma.

carry out investigations in dry farming areas, and an opportunity occurred in Pontotoc County, Oklahoma, where in September and early October there was an extensive outbreak of encephalitis in horses (Reeves, Mack, and Hammon, 1947). Because it was late in the season when the survey began, arthropod collections were small No virus was isolated from mosquitoes, horn flies, or chicken mites. Sera were obtained from wild and domestic animals on farms where equine encephalitis occurred. Only 15 out of 104 sera tested had antibodies to the western encephalitis virus, and 9 of these were from horses recovering from encephalitis, 8 out of 70 sera tested for St Louis virus were positive. Of chickens tested, only 2 out of 27 had neutralizing antibodies to the western virus, and none out of 22 to St. Louis virus

vectors

been ir

suga),

(*Dermanyssus gallinae*). Knowledge regarding vertebrate hosts is no more complete This report emphasizes the apparent differences between the epidemiology of these infections in the Far West and the dry farming areas of the East. In the latter areas, it may well be that the virus is adapted to a reservoir host other than birds, and that arthropods other than *C. tarsalis* are of importance.

PROPERTIES OF THE VIRUS

Physicochemical Properties

Size Experiments with Elford's collodion membranes have estimated the virus to be very small, probably from 20–35 mμ (Bauer, Cox, and Olitsky, 1935; Lazarus and Howitt, 1937, Tang, Elford, and Galloway, 1937). Calculations by Elford's centrifugation technique have confirmed these figures (Tang, Elford, and Galloway, 1937)

the electron microscope The easte ... -47 mμ (Taylor *et al.*, 1942 *a*, Shar ... or disk-shaped, and measures abo

in

194

cause maturation (McLimans, 1947).

on about 50 occasions. *Culex pipiens*, *Anopheles maculipennis freeborni*, and *Culiseta inornata* have also been found infected (Hammon *et al.*, 1941 *b*, 1942 *a*, *b*, 1945, Hammon and Reeves, 1945, 1947). There is little doubt that *Culex tarsalis* is the most important vector of equine and St. Louis encephalitis in the Yakima Valley This mosquito has been shown to feed frequently on domestic fowl and other animals and man. The feeding habits of this mosquito alone can explain the incidence of encephalitis and the distribution of antibodies in man and animals (Reeves and Hammon, 1944). Nevertheless, in 1944, when there were no cases of human encephalitis, the infection rate of *Culex tarsalis* with western virus was very high (Hammon and Reeves, 1947).

With regard to the feeding habits of possible mosquito vectors, it was found that western and St Louis viruses were neutralized by about 50 per cent. of sera from domestic fowl, by 20 per cent of sera from wild birds, by 35 per cent. of sera from horses, cows, goats, pigs, mules, and dogs; less commonly did the sera of wild mammals neutralize the virus (Hammon *et al.*, 1942 *c*, 1945).

These workers therefo[illegible] rary reservoirs f[illegible] in horses than [illegible] that a large proportion of horses becomes immune, the horse epizootic expends itself some weeks before the outbreak in man

The rôle of domestic fowls has been further strengthened by the finding that they develop viremia when suffering from an inapparent infection (Hammon, 1943), and that mosquitoes can transmit virus from fowl to fowl.

2. San Joaquin Valley.

Hammon, Reeves, and Galindo (1945 *a*) studied the epidemiology in this valley in Kern County, California, in 1943. They identified 19 cases of western infection and 5 cases of St. Louis encephalitis in man Over 10,000 blood-sucking arthropods were tested, and 31 strains of western virus were isolated, 28 strains of western virus were isolated from C. *tarsalis*, 2 from *Aedes dorsalis*, and 1 from *Culex stigmatosoma*. It was found that 55 per cent. of all C. *tarsalis* had engorged on birds, and chickens showed neutralizing antibodies to the western equine and St. Louis viruses. They concluded that in California, as in Yakima, *C. tarsalis* is the main vector. Some 622 C *tarsalis* caught in the winter yielded no virus.

A later paper (Hammon and Reeves, 1947) refers to 1943 as well as 1944; in the latter year there were only 2 human infections due to western and 3 to St Louis virus From 2,494 *Culex tarsalis* tested in 1944 one strain of western and one strain of California virus was isolated, from 4,425 *Aedes dorsalis* one strain of St Louis and one of California virus was isolated, these rates were very low compared to 1933. Again, chicken sera were found to neutralize western and St Louis viruses.

3. Canada.

In Manitoba in 1944 one strain of western virus was recovered from a pool of *Culex restuans* (Norris, 1946) Virus has also been found in *C. tarsalis* (McLintock, 1947).

4. Texas.

In the Lower Rio Grande Valley, Hammon, Reeves, and Irons (1944) could not isolate virus from 19 species of mosquito Antibodies were rare in the sera of horses and chickens.

5. Colorado.

Black, Absher, and McDonald (1945) investigated the mosquito situation in 1943–4 They found *C tarsalis*, *C pipiens*, and *Mansonia perturbans* the common

Eggs

Eggs can be infected by any route of inoculation, virus generalizing and killing

flammatory changes, thrombosis, and hemorrhage, appear a few hours after inoculation. The membrane becomes thickened and opaque The virus generalizes and invades the embryo, which dies after 16–24 hours, it is found in the amniotic fluid and blood (Covell, 1934, Higbie and Howitt, 1935; Bang, 1943). Multiplication begins 3 hours after inoculation, and reaches the maximum titer at 16–24 hours (Bang, 1943). Covell (1934) found acidophilic inclusions in the nuclei of the nerve cells in the brain, with some necrosis.

Virus-serum mixtures can be titrated on the membrane (Higbie and Howitt, 1935). The membrane can also be used in virus isolation work. Virus suspensions can also be titrated after inoculation on the membrane, using 50 per cent. embryo mortality end points (Bang, 1943).

It has been shown that the resistance of the embryo increases with increasing age, in older embryos death is delayed and the virus titers are lower (Bang, 1943)

Duffy (1944) has shown that the western virus grows only poorly if injected into embryos in which St. Louis virus has been previously inoculated.

The yolk sac route has been used (Cox, 1939, Stimpert, 1939). In these laboratories, Crawley has made extensive use of this route. Virus invades, and produces death of the embryo within 24 hours Virus suspensions and virus-serum mixtures can be titrated by this route, death of the embryo being taken as the end point (1948).

With the western strain, no evidence was obtained that serial egg passage altered antigenic structure (Shahan and Eichhorn, 1941).

Inoculation of the embryonated egg is widely used in the preparation of formolized vaccine from embryonic tissue (see p. 1131). Beard and coworkers have prepared purified virus from infected embryonic tissue (see above)

EXPERIMENTAL INFECTION OF ANIMALS

Horses

Horses can be infected by cerebral inoculation with either type In addition, the eastern virus infects by the intravenous and intracutaneous routes (Meyer, 1933 *b*, Meyer *et al*, 1934)

Infected horses show the following phases of illness (Ten Broeck, Hurst, and Traub, 1935, Mitchell, Walker, and McKercher, 1938) (1) there is an early stage of fever with viremia, (2) the animals then become hypersensitive, (3) tremors, neck rigidity, and slight lethargy are noted, (4) finally there is marked lethargy, tetanoid spasms, and Cheyne-Stokes breathing

The pathological changes in horses have been studied by Meyer (1933 *b*) and Hurst (1934) The changes, which are more intense and extensive with the eastern strain, are those of a primary nerve cell degeneration, with leukocytic infiltration Inclusions like those of Borna disease may be seen in the nuclei

Other Domestic Animals

The following are susceptible *goats*, either type (Meyer *et al*, 1934; Shahan, Giltner, and Schoening, 1939), *calves*, both types (Giltner and Shahan, 1933 *b*, Hurst, 1934, Shahan, Giltner, and Schoening, 1939), *sheep*, eastern virus (Hurst, 1934, Shahan, Giltner, and Schoening, 1939), *pigs*, both types (Shahan, Giltner, and Schoening, 1939), *dogs*, both types (Hurst, 1934, Shahan, Giltner, and Schoening, 1939, Howitt, 1940, Schlotthauer, 1941).

Infectivity (western virus) is said to be preserved in 50 per cent. glycerol (Howitt, 1934 *b*), or by rapid complete drying *in vacuo* over phosphorus pentoxide and storage *in vacuo* (Shahan and Eichhorn, 1941). Infectivity is well preserved in glass vials kept in the dry icebox.

Purified eastern virus is most stable at pH 7–10 (Finkelstein *et al.*, 1940).

Miscellaneous. Brain tissue infected with western virus shows metabolic differences from tissue infected with poliomyelitis (Nickle and Kabat, 1944). The western virus depresses the rate of anaerobic glycolysis of embryonic tissues (Victor and Huang, 1944). Rosenow claims to have isolated virus from streptococci (1942, Rosenow and Caldwell, 1941)

The Chemical Nature of Encephalomyelitis Virus

Wyckoff (1937) carried out ultracentrifugal analysis of infected tissue, and isolated a heavy protein-like component Later studies of an exhaustive nature, that can only be briefly summarized as follows, have been carried out by Beard and his associates (Finkelstein *et al.*, 1938; Sharp *et al*, 1939 *a*, *b*, 1940 *a*, *b*, 1943; Taylor *et al*, 1939, 1940 *a*, *b*, 1941 *a*, *b*, 1942 *b*, 1943; Taylor, Sharp, and Beard, 1940, see also *Ch. VII*).

1. They have worked with infected chick embryo tissue, and have purified the contained virus by ultracentrifugation.

2. A macromolecular infectious component has been isolated from infected embryos (both types of infection). The S_{20} of the eastern virus is 273×10^{-13}, and of the western virus 265.5×10^{-13}. Calculations of size give 50 4 mμ (eastern) and 56 8 mμ (western). These heavy components are most stable at pH 7–8 5. They are inactivated by ultraviolet light They behave as virus in infectivity experiments The infectious unit of eastern virus was thought to represent 250 molecules. The virus is a complex of high molecular weight, and contains phospholipids, cholesterol, fatty acid, and ribonucleoprotein.

3 Suitably purified chick embryo vaccines have been prepared, eliminating most of the unwanted protein material

4 A macromolecular lipoprotein complex containing 10 5 per cent ribonucleic acid can be isolated from normal embryos. It has a S_{20} of 78.7×10^{-13}, a molecular weight of 4,800,000, and a particle diameter of about 23 mμ A similar component in infected embryo tissue is unrelated to the virus

It may here be mentioned that another group of workers has found highly purified preparations of eastern virus to contain up to 77 per cent of components antigenically related to normal tissue components (Engel and Randall, 1947 *b*)

Reaction to Physical and Chemical Agents

Only a few special observations will be mentioned, the virus being fairly readily destroyed by physical and chemical agents Thus, Kempf *et al* (1945) found that the eastern virus was inactivated by a chlorine concentration of 0.71 p p m Both types are destroyed by ether (Birch, 1941, Sulkin and Zarafonetis, 1947) Mustard was found to inactivate the eastern virus, antigenicity being retained (Ten Broeck and Herriott, 1946) Aspergillic acid has no effect *in vivo* or *in vitro* on the infectivity of the western virus (Sulkin and Goth, 1945) Penicillin is ineffective in tissue cultures or eggs (Parker and Diefendorf, 1944)

Tissue Cultivation

The western virus has been cultivated in tissue culture (Olitsky, Cox, and Syverton, 1934, Zinsser and Schoenbach, 1937, Sanders and Molloy, 1940, Huang, 1943 *a*, Sanders and Huang, 1944) The eastern virus has also been cultivated (Cox, 1936)

creasing age a gradual decrease in susceptibility to infection by the peritoneal route, with increasing age there was a prolongation of the incubation period in those that did succumb. In young mice, virus was recovered in large amounts from the blood, but in adults only small amounts were found Lennette and Koprowski made similar observations with both strains They found that 3-day mice were susceptible to subcutaneous injection, intraperitoneally, mice up to 14 days of age were susceptible. In practice, age does not influence susceptibility to cerebral injection

Mice suffering from infection with the western virus were found to be more susceptible than usual to the dehydrating effect of intraperitoneal glucose (Hoyt, Holden, and Rawson, 1939) An intraperitoneal, intramuscular, or intravenous injection of glycerin or concentrated salt enhanced the activity of eastern virus injected intramuscularly, apparently due to dehydration (King, 1940 *a*, 1942) Etherization after exposure has been reported as exerting a beneficial effect on the course of western infection in mice, the effect on eastern infection being less marked (Sulkin, Zarafonetis, and Goth, 1945, 1946).

The pathogenesis of the infection has been studied after various routes of inoculation. After nasal inoculation (eastern virus) it appears that virus spreads by the olfactory nerves to the brain (Sabin, 1938)

After peritoneal inoculation in young mice, virus invades the blood (Olitsky, and Harford, 1938 *b*, Morgan, 1941), and probably reaches the CNS by being excreted in the nasal mucosa and then passing by the olfactory route

Sabin and Olitsky (1938 *a*) studied the effect of inoculating virus in the leg muscles With the eastern virus, in the majority of cases, virus entered the blood and passed to the brain by the olfactory paths, in a few cases, the virus spread by the nerves. With western virus in young mice, the blood and olfactory system were usually involved, in older animals spread was usually by nerve fibers

On intra-ocular injection, eastern virus was found to invade by the optic nerves (King, 1940 *c*).

[illegible]

Guinea-pigs

[illegible] the cerebral route produc[illegible] 1933 *b*) The virulence of [illegible]astern virus is the more virulent. Guinea-pigs are also susceptible by the following peripheral routes nasal, pad, ocular, muscular, peritoneal, and subcutaneous

A number of workers has investigated the pathogenesis and pathology of the infection after peripheral inoculation Thus, Howitt (1934 *a*) showed that when infected [illegible] virus [illegible] Remo[illegible] ing on nasal inoculation

Detailed studies have been carried out by Hurst (1936). After intramuscular injection, he showed that most animals infected with eastern virus developed nervous symptoms and died With the western virus, some survived and became immune He found that inapparent infections were unusual There was little evidence of local proliferation at the site of muscular inoculation, but increase was evident when eastern virus was injected in the pads

He found a phase of viremia, as in monkeys, and again virus was not found in any quantity in the viscera Multiplication of virus must have occurred in the blood or closely associated tissues After these routes of inoculation, virus was first found in the anterior brain, and not in the cord. Virus appeared to pass to the CNS indirectly from the blood, and not by direct neural paths.

Monkeys

Monkeys and apes can be infected by the cerebral and many peripheral routes, and die in a few days (Meyer, 1933 *b*, Hurst, 1936, Webster and Wright, 1938, Wyckoff and Tesar, 1939, Howitt, 1941 *b*).

Virus is found in the blood and cerebrospinal fluid. The peripheral routes used include: nasal, venous, muscular, dermal, and intralingual.

The most detailed investigations are those of Hurst (1936) who has studied the pathogenesis of the infection due to both types of virus, particularly after peripheral inoculation (i e, intradermal, intramuscular, intravenous, and intrasciatic) He has shown that the monkey exhibits 2 phases of illness. In the first, or visceral phase, virus circulates in the blood, and appears to multiply there, or in some tissue associated therewith, as no large quantity is found in the viscera. The only histological clew was afforded by the finding of occasional necrotic cells in the liver during this phase of viremia Animals inoculated peripherally may show no more than this phase. The second phase of nervous involvement may follow the visceral, *there is a rise of temperature, tremor, excitement, paralysis, or ataxia. This involvement* occurs at a time when there is a prompt antibody response, and disappearance of virus from the blood Unless there are signs of nervous involvement, recovered monkeys are not usually resistant to cerebral inoculation.

Virus may be found in the CSF before the onset of nervous symptoms. He found no evidence of neural spread after intradermal or intramuscular inoculation in the thigh, the anterior brain becoming infective before the cord The findings suggested that virus does not directly penetrate the blood brain barrier. In further support of the absence of neural spread, changes were only found late in the spinal ganglia.

Hurst (1936) found that the lesions in the nervous system of monkeys are similar to those in the horse, and appeared first at the time of rise of temperature After intradermal inoculation, there was some local cellular necrosis and inflammatory reaction.

Mice

Mice can be infected with the eastern and western viruses cerebrally and nasally. Under certain conditions, peripheral inoculation may secure infection, for example, subcutaneously, peritoneally, intramuscularly, intra-ocularly, into a nerve, in the pad, and intravenously.

Signs of illness appear in 48–72 hours, and the animals become rapidly prostrate and die shortly afterward The brain shows inflammatory and degenerative lesions (Olitsky, Cox, and Syverton, 1934) There is edema, congestion, focal hemorrhage and necrosis of Purkinje cells, and nerve cells of the motor nuclei in the brain stem and cord Intranuclear inclusions may be seen when the more chronic illness initiated by the nasal route is studied They are 1–2 μ in diameter, acidophilic, and occur in the hippocampus and anterior gray matter of the cord

The effect of age on susceptibility has been investigated (Olitsky, Cox, and Syverton, 1934, Olitsky and Harford, 1938 *a*, Sabin and Olitsky, 1938 *b*, King, 1940 *a*, *b*, Morgan, 1941, Lennette and Koprowski, 1944 *a*, *b*) Thus, Sabin and Olitsky (1938 *b*) found that with the eastern virus, 15-day mice were all susceptible by the muscular or peritoneal routes, at one month only 50 per cent could be infected, and at 3 months hardly any Resistant older animals could be infected by the sciatic nerve. With the western virus, 80–90 per cent of 15-day mice developed encephalitis after inoculation in the muscles of an extremity, at 21 days, the majority developed flaccid paralysis, and some resisted, at one month, almost all resisted Sciatic inoculation, however, produced paralysis

Infant mice are susceptible to peritoneal inoculation, but adults resist (Olitsky, Cox, and Syverton, 1934). Working with the eastern virus, Morgan found with in-

Birds

Various experiments on the infection of chickens have been referred to above (see Epidemiology). Fowls develop viremia on subcutaneous injection (Howitt, 1940, Tyzzer and Sellards, 1941)

Pigeons have been infected with the eastern virus (Giltner and Shahan, 1933 *b*; Traub and Ten Broeck, 1935, Fothergill and Dingle, 1938, Traub, 1938, Shahan, Giltner, and Schoening, 1939), and the western virus (Shahan, Giltner, and Schoening, 1939, Howitt, 1940, Graham and Levine, 1941, 1942).

Pheasants have been infected with the eastern strain (Shahan, Giltner, and Schoening, 1939, Sellards, Tyzzer, and Bennett, 1941).

The western virus can infect the duck, turkey, goose, hawk, guinea fowl, sparrow, quail, junco, thrasher, and vulture (Remlinger and Bailley, 1936 *a*, *e*, Shahan, Giltner, and Schoening, 1939, Howitt, 1940).

The burrowing or ground owl is susceptible to both types (Syverton and Berry, 1941 *a*).

ANTIGEN-ANTIBODY REACTIONS: EXPERIMENTAL CONSIDERATIONS

Virus Neutralization

Antibodies that neutralize the infectivity of virus rapidly develop in convalescence from infection in man and animals.

Antibodies also develop after the immunization of animals with formalin-inactivated virus, they may reach as high a titer as in animals vaccinated with live virus (see, e g, Cox and Olitsky, 1936 *b*, Morgan and Olitsky, 1941). Certain differences in the protective powers of sera obtained after immunization with live or formolized virus have been described (Olitsky and Harford, 1938 *c*, Olitsky and Morgan, 1939 *b*).

Working with eas [illegible] development of serum antibodies [illegible] nay be transmitted to young mic [illegible]

Ruchman (1946) [illegible] effect of diet on cerebral resistanc [illegible] isted 100–200 times as much virus cerebrally as did underfed mice. The sera of well fed mice neutralized between 10 to 100 times as much virus as did the sera of underfed animals Removal of the whole vitamin B complex from the diet produced no very definite results Deficiency of thiamin or riboflavin had no effect on the production of antibodies or cerebral resistance However, diets deficient in protein or carbohydrate lowered cerebral immunity to about 1/10 of that in controls

When serum antibody has fallen some months after the inoculation of live or formolized virus, a single dose of either type of antigen induces a greater antibody rise than occurs after the first inoculation (Morgan and Olitsky, 1941) Serum antibodies are associated with beta and gamma globulin (Koprowski, Richmond, and Moore, 1947)

Antibodies appear also in the CSF after vaccination with live or inactive virus (Howitt, 1941 *b*, Morgan, Schlesinger, and Olitsky, 1942)

Technique of tests.

Tests for virus neutralization can be carried out in various ways. A popular method involves the inoculation of serum-virus mixtures cerebrally in guinea-pigs.

A clew to the route of invasion of the CNS, after intradermal and intramuscular injection, was furnished by observations on the effect of nasal inoculation. Virus was found first in the olfactory bulbs and anterior frontal region. There was inflammatory infiltration of the bulbs and overlying meninges, and in the frontal brain meningitis, microglial proliferation, and neuronal destruction A similar distribution of lesions was sometimes found after muscular injection. He found in some cases during the period of viremia following peripheral inoculation (western) that virus could be detected in the nasal washings He suggested that in some cases at least, after intramuscular injection, the nervous system is invaded by virus which has passed from the blood into the nasal secretions.

It seems that after nasal inoculation, virus ascends via the perineural lymphatics of the olfactory nerves. After inoculation by other routes, virus may be excreted into the nasal passages, and ascend to the CNS by the olfactory route.

Peripheral routes of inoculation were also used by King (1938) with the eastern virus He found the earliest changes in the brain to take the form of a focal inflammation of the blood vessels. Before the onset of symptoms, such changes were *found irregularly in the gray matter, especially the neo- and olfactory cortices.* Neuronal destruction might also occur He found inflammatory changes to be less marked after cerebral inoculation.

He concluded (1939 *a*) that the frequency of lesions in the neocortex with intact subcortical centers rendered nerve spread improbable. Virus injected peripherally invaded the blood, and passed from there into the brain An exception was *the case of ocular injection where axonal paths of the optic nerves were involved* (1939 *b*).

Western virus transmitted serially in the pad eventually loses its neurotropic powers, and becomes dermotropic (Olitsky, Cox, and Syverton, 1934)

Guinea-pigs immunized with the western virus, and then injected cerebrally with this virus were found to be resistant to a subsequent cerebral injection of eastern virus, for about 2 weeks, or vesicular stomatitis virus for 4 days. This may be an example of the interference phenomenon, or may be due to a nonspecific neutralizing effect exerted by antibody appearing in the brain (Schlesinger, Olitsky, and Morgan, 1944).

Various Rodents

The following have been proved susceptible woodrat, both types of virus (Howitt, 1940; Grundmann *et al.*, 1943), cotton rat, both types (Howitt, 1940, Grundmann *et al*, 1943), field vole and woodchuck, both types (Syverton and Berry, 1940) [illegible] or gopher, both types (Syverton and Berry, 1936, [illegible] 40), wild mice, western virus (Howitt, 1940), rats, [illegible] chipmunks and ground hogs, western virus (Mitchell and Walker, 1941), hamsters, both types (Shahan and Creech, 1942, Watson and Smadel, 1943), hedgehog, eastern virus (Shahan, Giltner, and Schoening, 1939)

Rabbits

Rabbits can be infected cerebrally, in adult rabbits, subcutaneous injection may lead to inapparent invasion of the CNS, preceded by viremia, in young rabbits encephalitis follows subcutaneous injection (Meyer, 1933 *b*, Schlesinger, Morgan, and Olitsky, 1942).

Wild rabbits are said to be susceptible to both types (Howitt, 1940, Syverton and Berry, 1940).

If large doses of either virus are injected in the eye, corneal edema and opacity result, perhaps due to a toxic reaction (Evans and Bolin, 1946).

activated serum-treated virus suggested that aggregation occurred in the first set of conditions It appears that when serum acts on virus, both agglutination and neutralization take place. The phenomenon of "percentage neutralization" is probably dependent on aggregation (see also, Cox and Olitsky, 1936 *b*).

2 As occurs with other viruses, reactivation of neutral mixtures may occur on dilution (Pierce, Kempf, and Soule, 1941), but after prolonged contact this may not be possible (Labzoffsky, 1946 *b*).

[illegible]

vestigated (Cox and Olitsky, 1936 *b*, Olitsky and Harford, 1938 *a*; and others). Thus, incubation at 37° C. for 2½ hours did not alter the protective action of serum inoculated peritoneally in young mice (Olitsky and Harford, 1938 *a*). In work with the egg, Crawley has found that neutralization of eastern virus by antiserum occurs immediately, and is not increased by incubation

On the other hand, using western virus Morgan (1945) concluded that a reaction did occur between serum and virus on incubation at 37° C.

Making similar observations with the eastern virus, using cerebral inoculation in mice, Labzoffsky (1946 *b*) reported a progressive decrease in infectivity with increased duration of contact at 37° C.

5. If antibody does combine with virus in test tubes, the combination cannot be stable, for differing degrees of neutralization are demonstrated according as to whether the mixtures are inoculated cerebrally or peritoneally in young mice (Olitsky and Harford, 1938 *a*). In a second experiment (1938 *b*) using the passive immunity technique, they showed that this variation in protective capacity was correlated with the path followed by the virus to the CNS. When virus is inoculated peritoneally or muscularly, it invades the blood and neutralization presumably takes place there, or in some non-nervous tissue. When virus is inoculated in nerve tissue, however, little protection occurs.

6 A complement-like substance plays some part in the virus neutralization reaction. The neutralizing capacity of western virus antiserum falls off if kept at 4° C., and can be fully restored by the addition of complement. Neutralizing antibody in rabbit serum present in the pseudoglobulin fraction can be enhanced in effect by complement (Morgan, 1945). Whitman (1947) showed that the action of neutralizing antibody to western virus is augmented by the presence in normal serum of a heat-labile accessory substance This substance loses most of its activity when diluted 1/10, or when inactivated by heat, or when the serum is frozen at −20° C.

Complement Fixation

Complement fixation can be demonstrated readily between antisera and appropriate antigens

Although the complement fixing and virus neutralizing antibodies often run parallel (see, e g, Sulkin and Izumi, 1945), the fixation test may become negative while the neutralization test is still positive (Casals, 1945 *b*)

Antigens of various types have been used. Brain suspensions can be employed (Howitt, 1937 *b*) Extract prepared by alternate freezing and thawing can serve as antigen (Casals and Palacios, 1941 *a*, *c*). Irradiation of such suspensions produces a noninfective [illegible]

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Study has been made of the effect of using *young susceptible mice, injected* peripherally. Thus virus-serum mixtures given peritoneally were found to protect young mice in dilutions which induced infection by the cerebral route (Olitsky and Harford, 1938 *a*). Wright (1942) used an intraperitoneal protection test in unweaned mice. Lennette and Koprowski (1944 *b*) found that the younger the animal, the greater the amount of neutralization demonstrated. They used 3-day mice injected subcutaneously, or 8–14 day mice injected peritoneally. They showed that the cerebral route is too exacting, by comparing the results of inoculating human antiserum-virus mixtures subcutaneously in 3-day mice, and cerebrally in 28-day animals (1946). By the cerebral route, hardly any antibody was detected, but it was shown to be present in considerable amount by the subcutaneous route.

The stock of virus, to be of uniform potency, should be preserved frozen (see, e g, Hammon and Izumi, 1942)

Passive immunity.

High titer neutralizing antiserum can be produced by the immunization of *horses, rabbits, or guinea-pigs, but prolonged courses of injections are not needed*

Passive protection with serum can be shown experimentally. Thus with guinea-pigs, intravenous injection protects against a cerebral dose (Friedemann, Hollander, and Bornstein, 1944). Intracardial injection protects against an inoculation in the pad for 24–48 hours, in some cases, death is delayed (Olitsky, Schlesinger, and Morgan, 1943). Zichis and Shaughnessy (1945) also reported successful results in guinea-pigs.

Experiments of Olitsky and Harford (1938 *b*) may throw light on the mechanism involved in passive immunity in mice. They gave 12–15 day mice serum intraperitoneally, and then injected virus in the brain, nose, muscles, or abdomen Protection was only shown to the latter 2 routes of infection This is presumably because in these instances virus invades the blood and the site of neutralization is either here or in some non-nervous tissue When the pathway of the virus is in nervous tissue *from the beginning, there is little opportunity for neutralization*

As regards treatment, serum is said to arrest the infection in guinea-pigs and mice (Zichis and Shaughnessy, 1940) In monkeys, however, serum given at the *time of onset of symptoms* has not proved effective (Wyckoff and Tesar, 1939).

Significance of antibody.

The observations are somewhat conflicting. Thus, certain observers have found that the degree of resistance to infection in vaccinated mice correlates fairly well with serum antibody titer (Morgan, 1941, Morgan and Olitsky, 1941, Casals, 1943 *b*).

In rabbits, however, the presence of antibody in the CSF seemed *to be the* determining factor. Thus, if no antibody was found in the CSF, even if it was in the blood, animals succumbed to challenge (Morgan, Schlesinger and Olitsky, 1942, *Schlesinger, Morgan, and Olitsky, 1942*).

In vaccinated guinea-pigs, there may be a high degree of cerebral resistance, without much serum antibody being present (Olitsky and Harford, 1938 *c*)

***In vitro* interaction.**

There has been considerable discussion, and the position is still not clarified regarding what occurs when virus and antiserum are mixed *in vitro;* is there a stable union between virus and antibody? In the present state of knowledge, all we can do is to present the main observations, without drawing any definite conclusions.

1. Merrill (1936 *a, b*), working with eastern virus, found differences between the titers when *serum* was added to neat virus, and serial dilutions were then made, and the titers obtained when the virus and serum were mixed after being diluted separately. The titer was higher by the latter method Filtration tests of partly in-

can be immunized to resist a cerebral challenge. The degree of resistance is as high as occurs after the use of live virus (Cox and Olitsky, 1936 *a*, *c*, Beard *et al.*, 1938 *a*, Olitsky and Morgan, 1939 *b*, Morgan and Olitsky, 1941)

It is said to be easier to immunize older than younger mice sufficiently to withstand a cerebral challenge (Morgan, 1939, 1940, 1941).

Resistance induced by formolized virus wanes in the course of months, but a "booster" dose quickly raises the degree of immunity (Morgan and Olitsky, 1941)

Some work has been carried out with a formolized horse brain vaccine in the prevention of the disease in horses (Shahan and Giltner, 1935 *b*)

After prolonged mouse passage, strains may lose some of their antigenic properties (Olitsky, Morgan, and Schlesinger, 1945).

Chick embryo vaccines.

The vaccine most widely used for the immunization of horses is prepared from infected chick embryos, and formolized It can be used in the monovalent or bivalent form (Beard *et al*, 1938 *b*, Randall, 1940, Randall, Mills, and Engel, 1947). Experimentally, this vaccine induces satisfactory resistance in guinea-pigs (Lyon and Wyckoff, 1938, Mitchell, Walker, and Plummer, 1938, Schoening, 1939).

In horses, the vaccine is given in 2 doses at weekly intervals, intradermally (1 c.c.) or subcutaneously (10 c c), this dosage immunizes sufficiently to withstand a cerebral challenge for about a year (Eichhorn and Wyckoff, 1938, Lyon, 1938, Schoening, 1939, Roberts and Moore, 1939, Schoening *et al*, 1940, 1943; Simms, 1946). The vaccine has been widely used since 1938, and appears to have been re- [illegible] mple, experiences [illegible] (1945) reported [illegible] compared to 2 9 per 1,000 in unvaccinated animals.

Embryo vaccines can sensitize guinea-pigs, owing principally to antigens in egg white, ovalbumin, egg yolk, and of chicken origin (Coulson and Stevens, 1946, Stull, 1946, Engel and Randall, 1947 *a*). It is probable that formalin reduces somewhat the danger of anaphylactic reactions (Randall, Mills, and Engel, 1947). Nevertheless, anaphylactic reactions have occurred in horses (Wolfe and Trum, 1940).

Other vaccines.

Formolized tissue cultures can be used (Sanders and Molloy, 1940) Brain or chick embryo vaccines retain immunizing potency after exposure to UVL (Morgan and Lavin, 1941)

Mechanism of Resistance in Vaccinated Animals

There is little doubt that antibody present in the CSF or blood plays some part in the resistance shown by immunized animals, and this point has been discussed above The rôle of serum antibody is presumably restricted to infections in which virus reaches the CNS via the blood

There may be some part played by an acquired resistance of the nerve cells Thus, injecting western virus in vaccinated guinea-pigs, Schlesinger, Olitsky, and Morgan (1944) found a rise of temperature and recovery without signs of nervous involvement Histological changes were present, but virus did not multiply. Hurst (see above) found that immunity developed only if there had been signs of nervous involvement

Active Immunization in Man

The inoculation of chick embryo vaccine stimulates the development of virus neutralizing antibodies, but probably not in every case (Bowman, 1945, Heath, 1945) Beard, Beard, and Finkelstein (1939, 1940) used a bivalent vaccine. The response of neutralizing antibodies was higher and developed more rapidly with the

Specific antigens have been prepared from chick embryo tissue or mouse brain by extraction in the lyophilized state with benzene, toluene, or dichlorethylene (DeBoer and Cox, 1947). Espana and Hammon (1947) have modified this technique, and prepared antigens for the N. American equine viruses, and for Hammon's California virus.

Hyperimmune serum is often prepared in guinea-pigs. It is recommended that the virus be given subcutaneously, then peritoneally, and finally cerebrally (Hammon and Espana, 1947); with Hammon's California virus an injection is given cerebrally. Sera should normally be inactivated at 60° C. for 20 minutes, but if the serum is anticomplementary or if nonspecific fixation occurs, it should be heated at 65° C for 20 minutes (Casals, 1945 *b*).

Using irradiated chick embryo antigens, Brown (1947 *a*) found nonspecific fixation with some human sera. By inactivating the sera at 60° C. for 15 minutes instead of 56° C. for 30 minutes, this trouble was obviated.

Miscellaneous

Antisera do not agglutinate collodion particles mixed with virus (Donaldson and Clark, 1945).

SERUM ANTIBODIES IN MAN

Virus neutralizing and complement fixing antibodies develop in convalescence, and a distinct rise in titer should be evident about 10–14 days after onset (see, e g, Casals and Palacios, 1941 *b*). CF antibodies have been found as long as 2 to 2½ years after the illness (Casals and Palacios, 1941 *b*, Howitt, 1943).

There is little doubt that in endemic areas, subclinical immunization occurs A number of workers has found virus neutralizing antibodies in the sera of persons without any definite history of encephalitis (Olitsky and Morgan, 1939 *a*, Mitchell and Pullin, 1943, Bowman, 1945). Hammon and Reeves (1947) give the following figures for the incidence of antibodies in the general population to the western virus in Yakima 1940, 5 out of 75 (6 6 per cent) positive, 1941, 0/52 positive, 1944, 7/24 (29 3 per cent) positive It is of interest that in 1940 there were 58 clinical cases of encephalitis, in 1941 26 cases, and no cases occurred in 1944 Antibodies have not been found in the sera of persons in nonendemic areas, e g, in Quebec and Ontario (Mitchell and Pullin, 1943)

Antibodies may occur in the CSF (Howitt, 1941 *b*, Gwatkin and Moynihan, 1943)

ACTIVE IMMUNIZATION IN ANIMALS

With Live Virus

Animals that survive experimental infection are resistant to challenge, even by the cerebral route This phenomenon can be shown especially with horses, monkeys, and guinea-pigs

Immunity may also be induced by the injection of sublethal doses of virus Thus, in horses, the injection of virus subcutaneously may immunize (Records and Vawter, 1934 *a*) Working with guinea-pigs, Olitsky and Cox (1936) induced immunity by sublethal doses Howitt (1940) made similar observations in birds, and Casals (1943 *b*) in mice

Resistance waning after a few months is quickly reinforced by a booster dose (Morgan and Olitsky, 1941)

With Inactivated Virus

Formolized tissue vaccines.

Formolized animal brain tissue, presumably containing only inactivated virus, or too little live virus to be effective, is a potent antigen, and laboratory animals

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western than the eastern virus, high titers being found after 7 and 14 days, respectively. After 6–9 months, most of the eastern antibodies disappear, but western antibodies remain at a high level (Beard, Finkelstein, and Beard, 1941). These workers found that the response to revaccination with a single dose was markedly higher than that following primary vaccination.

Serum antibodies were titrated by Lennette and Koprowski (1946) by the sensitive subcutaneous route in 3-day mice. Although titration by the cerebral route demonstrated little antibody response, by the other method neutralizing antibodies were found by the 3rd week after the 1st dose The peak response was 1–5 weeks after the second injection, and then fell rapidly to reach a low level 9–10 months later.

Despite theoretical possibilities (see Ch. XIII), there does not seem to be much risk of specific sensitivity developing in man (Gold and Hampil, 1940–1).

RELATIONSHIP TO OTHER VIRUSES

The western and eastern viruses have been shown to be unrelated to the following neurotropic viruses. St. Louis, Japanese B, West Nile, Russian spring-summer, or louping ill (Howitt, 1937 *b*, Shahan *et al.*, 1942; Casals, 1944), lymphocytic choriomeningitis (Howitt, 1937 *a*, *b*), vesicular stomatitis (Olitsky, Cox and Syverton, 1934), infectious encephalomyelitis of young chickens (Olitsky, 1939), and Moscow 2 virus, which is a rabies strain (Howitt, 1937 *a*, 1941 *a*).

The N. American strains are unrelated to the following viruses. infectious feline agranulocytosis (Lawrence *et al.*, 1943), Bunyamwera (Smithburn, Haddow, and Mahaffy, 1946), Roca-García's (1944) viruses, Colorado tick fever (Koprowski and Cox, 1947).

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CHAPTER XCVII

VENEZUELAN EQUINE ENCEPHALOMYELITIS

EQUINE encephalomyelitis in Venezuela is caused by a virus that is similar in properties to the eastern type of the North American virus (Kubeš and Ríos, 1939, Gilyard, 1945) The Venezuelan virus is, however, antigenically distinct from the N American strains, and from the Argentine virus (Beck and Wyckoff, 1938, Kubeš and Ríos, 1939, Kubeš and Diamante, 1942).

The virus probably also occurs in equines in Colombia (Soriano Lleras and Figueroa, 1942; Samper and Soriano Lleras, 1943)

It has been recovered from horses and mules in Trinidad, where in 1943 there were about 70 fatal equine cases (Kubes, 1944, Randall and Mills, 1944).

A natural case of infection in man has been reported from Trinidad (Gilyard, 1944, Randall and Mills, 1944) The virus has been recovered from the blood and upper respiratory tract of a laboratory worker suffering from a mild acute febrile illness (Casals, Curnen, and Thomas, 1943), the same workers have described an-
[illegible] have been described by Lennette and
[illegible] Cox (1947 *a*), which serves to em-
[illegible] nplement fixing and virus neutralizing
[illegible] Curnen, and Thomas, 1943) Gallia
and Kubeš (1944) described the presence of virus neutralizing antibodies in the sera of a number of laboratory workers who probably contracted an inapparent infection.

It seems very likely that the infection is borne by mosquitoes, and the virus has been transmitted to guinea-pigs by *Aedes aegypti*, *A. albopictus*, and *A geniculatus* (Roubaud *et al*, 1941). Virus was recovered in Trinidad from *Mansonia titillans* naturally infected, and this species was shown to be infective by bite (Gilyard, 1944)

The virus is pathogenic experimentally for horses, guinea-pigs, rabbits, and mice (Beck and Wyckoff, 1938, Kubeš and Ríos, 1939, Kubeš, 1944, Koprowski and Lennette, 1946 *a*) Mice of all ages are highly susceptible to extraneural inoculation
(Lennette and [illegible]
as to cerebral [illegible]
not significantly [illegible]
from the N A [illegible]
formed by the peritoneal route in mice up to 200 days of age (Lennette and Koprowski, 1944 *b*)

In the egg, the virus can be inoculated in the yolk sac or on the chorio-allantoic membrane (Kubeš and Ríos, 1939, Kubeš, 1944). Virus multiplies rapidly, and disseminates throughout the egg, reaching its highest concentration in the embryo Virus can be titrated by egg inoculation, and the results are similar to those obtained in mice (Koprowski and Lennette, 1944)

The virus has been grown in tissue culture, in serum-Tyrode and minced chick embryo (minus the CNS). Studying the effect of continued culture passage on invasiveness, the lethal effect of the virus on cerebral inoculation in mice was found not to be affected by 74 passages but an effect was noted on peripheral inoculation The first indication that virulence by the extraneural route was being lost on passage was obtained in older mice (28 days and upward) After repeated culture [illegible]

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CHAPTER XCVIII

RUSSIAN SPRING-SUMMER (FAR EASTERN) ENCEPHALITIS

In the past ten years or so, Russian workers have thoroughly investigated a type of tick-borne encephalitis occurring mainly in the virgin woodlands (*taiga*) of the far eastern part of the Soviet. It is probable that the disease existed as far back as 1914, and perhaps earlier, although it only became a problem in about 1935.

More recently, a western form of the disease has been recognized in European Russia.

Most of th[illegible] of useful articles and [illegible] 940, 1941, Propper-Gra[illegible] 940; Solo-viev, 1944; S[illegible] Particular mention mus[illegible] printed in the *Bulletin of Hygiene*, to which suitable acknowledgment is made.

In the compilation of this article, we have freely consulted the valuable reviews of Smorodintsev (1940) and Silber and Soloviev (1946). (As we have not been in a position to consult or read any Russian language papers, we have to depart from our usual practice as regards references, and can only mention English language articles in which the appropriate original Russian references are quoted. Silber and Soloviev (1946) give a full Russian bibliography.)

CLINICAL FEATURES

The incubation period of far eastern encephalitis is 8–18 days. The onset is sudden, with rise of temperature, head[illegible] pains in the neck T[illegible] sis may involve the [illegible] sleepy, and may have epileptifo[illegible] convulsions. Various types of temperature reaction have been described. In severe cases, the bulb is involved with consequent paralysis of vital functions.

There is a slight increase in lymphocytes in the CSF, and an increase in protein.

Sequelae include paralysis and atrophy of the muscles of the neck and brachial girdle. Parkinsonism is said not to occur.

The mortality of the eastern form is about 30 per cent, death occurring 3–8 days after onset.

In some cases, the picture is more of a serous meningitis than an encephalitis.

Cases of the western type occurring in European Russia tend to be milder, and abortive forms are more common.

DISTRIBUTION OF VIRUS

Apart from the tissues of the central nervous system, virus can be found in the spinal fluid, blood, liver, spleen, urine, nose, and throat (see Smorodintsev, 1940, Silber and Soloviev, 1946). It has also been recovered from the pharynx of contacts.

PATHOLOGICAL FEATURES

The features are those of an acute nonsuppurative meningo-polio-encephalomyelitis (Smorodintsev, 1940, Silber and Soloviev, 1946). The meninges are inflamed, being infiltrated with round and polymorph cells. The brain is congested, and there are numerous hemorrhages in the brain stem, medulla, and spinal cord.

the order of sensitivity (Koprowski and Lennette, 1946 *b*). first, intraperitoneal inoculation in mice; and then of equal sensitivity come intracerebral inoculation in mice and inoculation in chick embryos Complement fixation can also be demonstrated (Casals, 1945, 1947).

Guinea-pigs can be actively immunized with formolized virus (Beck and Wyckoff, 1938), and horses have been vaccinated with chick embryo vaccine (Kubeš and Ríos, 1938). Hyperimmune sera can be prepared in rabbits by injection of formolized followed by live virus (Koprowski and Lennette, 1946 *b*).

The virus is not related antigenically to the virus of Colorado tick fever (Koprowski and Cox, 1947 *b*).

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tine (Pavlovsky and Soloviev, 1940). Transmission of infection to man or animal is by salivary contamination of the bite.

In laboratory experiments, *Ixodes persulcatus* can be infected by feeding on mice (Pavlovsky and Soloviev, 1940). *Ixodes persulcatus* is thought to be the only tick transmitting the far eastern form of the disease.

Ixodes ricinus. A virus thought to be that of spring-summer encephalitis has been isolated from larvae and nymphs of *Ixodes ricinus* in White Russia and Karelia, and this tick has been held to be the vector of the western form of the disease (Chumakov and Naidenova, 1944; Chumakov *et al*, 1944). Silber and Shubladze (1945 *b*), however, raise the question of possible confusion with the virus of louping ill Silber and Soloviev (1946) conclude that the epidemiology of the western form of the disease is insufficiently studied, and any conclusions as to its method of spread are premature

Other ticks. In laboratory experiments, of unknown practical application, virus has been found to survive in the following ticks *Dermacentor silvarum, D pictus, D nuttalli, Rhipicephalus turanicus, Rh. bursa, Haemaphysalis concinna, Hyalomma asiaticum, H. dromedarium, H. turkmeniense;* transovarial infection has been noted in *D. nuttalli, Rh. turanicus, H asiaticum, H turkmeniense,* and *H. dromedarium* (Smorodintsev, 1940, Chumakov, Petrova, and Sondak, 1945). *Ornithodoros moubata* has also been infected (Parker, 1942).

Persistence of virus in ticks. The question arises of whether virus can survive in the tick population over the severe Russian winter. There is little doubt that it can do so, and that ticks constitute an important, probably the main, reservoir of infection with the far eastern virus Thus, it has been shown that once acquired, virus is transmitted to successive stages in metamorphosis, and transovarially. Although it has been found that eggs and unfed larvae perish at –7° C, all other stages can survive down to –30° C, which temperature is not reached under the snow covering (Pshenichnov and Khramushen, 1943) Demonstrating the persistence of virus in laboratory experiments with *I ricinus*, Chumakov (1944), found that it could be recovered from nymphs of the 2nd generation 11 months after collection from a 2nd generation imago after 15–18 months, and from larvae and nymphs of the 3rd generation after 22–26 months Transovarial transfer of virus was noted through 3 generations

Prevention of tick bites Ticks are found in the lower zones of vegetation, not higher than 1 meter from the ground. They can be kept away from man by protective clothing tied tightly at the ankles, wrists, and neck, and by chemically impregnated garments (Eskin, Chagin, and Murovannyi, 1944)

Animal hosts of ticks Ticks feed on many animals, and man is only an accidental host No less than 63 species of mammal may act as host, but the hosts of the adult of *Ixodes* are only 25, mostly wild carnivores, ungulates, dogs, pigs, cattle, sheep, horses, and goats (Kuzyakin, 1942)

Numerous animals have been found infected, either by isolation of virus, or by demonstration of serum antibodies (Chumakov, Vorobieva, and Sofronova, 1940, Smorodintsev, 1940, Pavlovsky, 1941, Soloviev, 1941, 1944, Kalabukhov and Shubladze, 1946) The following have been incriminated hare, squirrel, chipmunk, red-backed mouse, hedgehog, vole, mole, water rat, wild and wood mouse, hazel grouse, and other birds

The virus has been isolated from the brains of cows killed near Brest-Litovsk, and antibodies have been found in the sera of bovines in this district (Chumakov and Naidenova, 1944)

Animals obviously play an important rôle in the epidemiology of Russian encephalitis, as they serve to feed the tick vectors Animals almost certainly disseminate infected ticks over a wide area, but it is doubtful whether they constitute an important reservoir of infection The period of viremia is short, and, therefore, animals can be infective for ticks for only a brief period

The severest lesions occur in the gray matter of the cord and medulla, changes are less severe in the gray matter of the base of the brain, and are slight in the cortex. The types of lesion seen in the various areas include acute perivascular infiltration, vasculitis and hyaline thrombosis, a diffuse and focal infiltration of the gray matter; degenerative changes in the ganglionic cells of the gray matter of the medulla and cord, progressing as far as complete destruction and neuronophagia, the infiltrating cells are mostly lymphocytes, with a few polymorphs and glial cells The changes are likened to those of St. Louis and Japanese encephalitis.

EPIDEMIOLOGY

Geographical Distribution

The disease occurs mainly in Siberia and the Far East of Russia, but also in th Urals, the pre-Urals, Volga, Karelia, White Russia, Kuibishev, Leningrad and Kalinsky (see Soloviev, 1944, Kalabukhov and Shubladze, 1946). A distinction is made between far eastern encephalitis and the milder western variety found in central and western Russia. In European Russia, it is probable that many cases diagnosed as atypical poliomyelitis, serous meningitis, and "post-grippe" encephalitis have really been due to invasion by the encephalitis virus (Chumakov and Seitlenok, 1940).

Seasonal Incidence

The majority of far eastern cases occurs in the last 10 days of May and the first 10 of June, the whole season extends from the end of April to August. The western cases occur mainly in the summer.

Type of Locality and Worker Involved

The far eastern disease occurs chiefly in the *taiga* or uncleared virgin forest The main incidence is in recently built villages in these forests. The chief victims are the young male lumber workers The incidence in recently cleared relatively uninhabited *taiga* is twice as high as in the more colonized parts

Rôle of Ticks [1]

The occurrence of cases in those who work in the *taiga* suggests that the vector of the far eastern disease may be an arthropod, and up to 75 per cent of cases actually have a history of tick bite within the incubation period. The curve of the incidence of ticks is almost exactly the same as that of the disease, but 10–14 days earlier

Ixodes persulcatus This tick is the vector of the disease *in the far eastern Soviet*. Virus has been recovered from ticks by inoculating mice, or by allowing them to feed on mice. The tick can absorb virus at any stage of its metamorphosis by feeding on an infected animal, once acquired, virus is transmitted to the subsequent developmental phases, and to the offspring transovarially. Virus has been recovered from larvae, nymphs, male and female adults, and hibernating adults (before feeding) collected in endemic foci (Levkovich and Skrynnyk, 1940, Smorodintsev, 1940, Pavlovsky, 1941, Ryzhov and Skrynnyk, 1941, Chumakov *et al*, 1944) Virus is present in all organs of infected ticks, especially the brain, sex organs, and intes-

[1] For the benefit of those who are not familiar with the subject, the biology of ticks is briefly as follows (see Whittick, 1943) Mating between adults occurs on the host [illegible] s, [illegible] in [illegible] ng
8-legged nymph. [illegible]
rise to the adult Mating occurs on the host and the cycle renews

IMMUNITY MECHANISMS

Experimental

Active immunity.

Mice that recover from infection become resistant, inoculation of a sublethal dose also immunizes (Soloviev, 1944).

Mice can be actively immunized by intraperitoneal injection of formol-inactivated virus (e.g., 10 per cent brain, 0.5 per cent. formol), mice so treated will resist a challenge dose of live virus injected by a peripheral route (Levkovich and Smorodintsev, 1940, Smorodintsev, 1940, *et al.*, 1941; Casals and Olitsky, 1945).

Serum antibodies.

Complement fixation can be demonstrated between antisera and brain antigens, the presence of a positive fixation test in the sera of immunized mice indicates a high degree of resistance to challenge (Casals, 1944; Casals and Olitsky, 1945): Casals (1947) gives details of his technique of preparing antigen by alternate freezing and thawing, inactivation is by exposure to UVL or heat. Antisera are readily prepared in guinea-pigs recovered from a cerebral inoculation (Hammon and España, 1947).

Virus neutralizing antibodies develop in mice on recovery from infection or after injection of formolized virus. These antibodies can be tested by injecting virus-serum mixtures peritoneally in mice, less antibody is demonstrated if mixtures are inoculated cerebrally (Smorodintsev *et al.*, 1941; Casals and Olitsky, 1945) Olitsky and Casals (1947) describe in detail the technique of virus neutralization tests using the cerebral route (see Ch. XI).

Virus neutralizing antibodies have been demonstrated in the serum of infected monkeys and hyperimmune rabbits (Silber and Shubladze, 1945 *a*, Silber and Soloviev, 1946), and hyperimmune goat serum has been used in man (see below).

Nonspecific virus neutralization has been demonstrated, and the lipoid fraction of normal animal serum rapidly reduces the infectivity of suspensions of Russian spring-summer virus (Casals and Olitsky, 1947)

As regards the *mechanism of resistance* in immune mice, it is probable that antibody plays an important rôle Casals and Olitsky (1945) found that the degree of resistance correlated with the presence and titer of neutralizing antibodies, if these were estimated by the peritoneal route in mice Further, there was a drop in titer of antibody following the inoculation of virus.

Passive immunity.

Antiserum exerts a definite protective effect in infected mice Thus mice given serum 1–15 days before infection may survive, serum may be administered up to one day after infection (Chumakov, 1940).

In Man

Dwellers in endemic areas acquire an immunity, presumably by subclinical attack The incidence of the disease in newcomers to the *taiga* is about 2½ times that in older residents (Soloviev, 1944, Silber and Soloviev, 1946).

Man can be immunized by the subcutaneous injection of formol-inactivated virus, and good results have been obtained in endemic foci, the incidence of the disease being reduced by over ten times (Smorodintsev, 1940, Smorodintsev, Levkovich, and Dankovsky, 1940, Smorodintsev *et al*, 1941)

Virus neutralizing antibodies develop in man on recovery from infection, and can be demonstrated after 2–4 weeks, reaching a maximum after 2–3 months (Chumakov, 1940, Smorodintsev, 1940, Silber and Soloviev, 1946).

PROPERTIES OF THE VIRUS

Antigenic Structure

So far, no evidence has been brought forward regarding antigenic heterogeneity, and it appears that all strains are similar in respect to antigenic structure (Smorodintsev, 1940; Silber and Soloviev, 1946).

Infection in Experimental Animals

Mice.

Virus can be isolated from human material most readily by cerebral injection in mice. Mice can also be infected nasall [illegible] and *subcutaneously (Smorodintsev, 1940, Casals* [illegible] 1946). Symptoms develop after about 4–10 [illegible] tive and later sluggish, with paralysis of the hind quarters Virus is found in the blood shortly after inoculation, and again in the terminal phase. It can be found in the urine from 3–6 days after infection. The histological appearances resemble those found in man.

Strains of mice bred selectively for susceptibility and resistance to louping ill and St. Louis viruses proved similarly susceptible and resistant to Russian virus (Casals and Schneider, 1943).

Monkeys.

On cerebral injection, encephalitis develops in 5–9 days, from which the animals may recover (Smorodintsev, 1940, Silber and Shubladze, 1945 *a*, Silber and Soloviev, 1946) They show fever and paralysis

Sheep.

Lambs have been infected by the cerebral route (Smorodintsev, 1940, Silber and Soloviev, 1946) They develop paralysis and fever, and die in about 7–10 days *Histologically, in addition to inflammatory changes, the brain shows some necrosis* of purkinje cells.

Other animals.

A number of other animals may harbor virus after injection the wild mouse, gray rat, goat, wolf, hedgehog, oriental water rat, hamster, and various birds—siskin, redpoll, goldfinch, and red breast (Smorodintsev, 1940, Soloviev, 1944, Silber and Soloviev, 1946). Guinea-pigs develop only slight fever on cerebral injection (Hammon and Espana, 1947)

Some Other Properties

The virus is grown in minced chick embryo in Tyrode's solution (Smorodintsev, 1940)

The virus passes through Seitz pads, Berkefeld V, N, and W candles, and Chamberland L_2, L_3, and L_5 filters (Smorodintsev, 1940, Silber and Soloviev, 1946) It is preserved in glycerol (Smorodintsev, 1940), and maintains infectivity when frozen and dried It is destroyed at 60° C in 10 minutes, and by 1 per cent phenol (Silber and Soloviev, 1946) Formol (1 500–1,750) destroys the virus in a 1 per cent. brain emulsion at ice box temperature in 20 days, at room temperature in 6 days, and at 37° C in 3 days (Levkovich and Smorodintsev, 1940, Smorodintsev *et al*, 1941).

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Antibodies can be demonstrated in the sera of residents in endemic areas (Silber and Soloviev, 1946).

Serum from hyperimmune animals (goats) or convalescent human beings is a useful therapeutic agent in man, if given intrathecally and intramuscularly early and often (Smorodintsev, 1940). It may also be used prophylactically in the case of persons staying only a short time in an endemic area (Chumakov, 1940).

RELATIONSHIP TO OTHER VIRUSES

Louping ill Russian virus is closely related antigenically to that of louping ill, as shown by complement fixation, neutralization, and intraperitoneal cross resistance tests in mice (Casals and Webster, 1943, 1944; Casals, 1944). Sheep are sensitive to both viruses, but whereas the Russian virus produces fatal paralysis, louping ill causes ataxia and tremor (Silber and Soloviev, 1946).

Japanese, St. Louis, and West Nile viruses. Here the results are somewhat conflicting. Thus, Casals (1944) found that the subgroup represented by these 3 viruses was unrelated to Russian or louping ill viruses. Russian observers, however, claim that Russian and louping ill viruses are related to Japanese and St. Louis viruses. Thus Levkovich (1940) showed that animals vaccinated with Russian virus were resistant to challenge with Japanese and St. Louis viruses. It has been claimed that Japanese sera exert a weak neutralizing effect on Russian virus. Russian sera were found to have a strong neutralizing effect on Japanese virus, but only a weak action on St. Louis virus Russian virus could completely absorb antibodies from Japanese serum, but Japanese virus could only partly absorb antibodies (Smorodintsev, 1940, Silber and Soloviev, 1946).

Other viruses Russian virus is not related to equine encephalomyelitis virus (Casals, 1944), and it is not neutralized by the sera of convalescents from encephalitis lethargica (Smorodintsev, 1940). It is not related to the virus of another tick-borne disease, Colorado tick fever (Koprowski and Cox, 1947)

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perivascular cuffing, there is a great diminution in the numbers of Purkinje cells, and neuronophagia and reactionary gliosis may be present in varying degree.

There may be perivascular cuffing, nerve cell destruction, and neuronophagia in the medulla and spinal cord also, but these are very variable features. The essential lesion in the naturally occurring disease is, therefore, the great destruction of nerve cells, more particularly the Purkinje cells of the cerebellum Here it may be mentioned, in anticipation, that similar appearances are found in the experimentally infected monkey. The mouse and pig, however, show much less evidence of cerebellar nerve cell damage

Epizootology

Distribution of the disease Louping ill occurs mainly in lambs and yearlings, but the highest death rate is among rams bought in for breeding purposes The disease occurs in the West Highlands and border districts of England and Scotland The affected farms have usually rough hill grazing and are infested with ticks The main louping ill season is from mid-March to mid-May with, in the West Highlands, an autumn outbreak as well

Method of transmission The work of the Moredun investigators (e g, Gordon *et al*, 1932 *a*; MacLeod and Gordon, 1932) has conclusively proved what had long been suspected, namely, the rôle of the tick *Ixodes ricinus* in the spread of louping ill The following are the main points elicited (1) the virus of louping ill can be recovered from ticks that have fed on sheep during the prodromal febrile period, (2) the virus can be demonstrated in the tissues of nymphs which, as larvae, have fed on infected sheep, (3) the infection can be transmitted to sheep by the bites of female and nymphal ticks which have acquired the infection respectively as nymphs and larvae, (4) ticks are capable of producing louping ill within 12 days of molting, or as soon as they are able to attack A publication (*Bulletin*, 1938) describes the life history and economic significance of ticks

The influence of tick-borne fever

It has already been mentioned that many sheep infected with louping ill show no more than the prodromal febrile reaction and do not develop symptoms referable to the central nervous system For the development of these symptoms the coexistence in the tick-infested sheep of a second disease, tick-borne fever, is a most important factor Cross immunity experiments have shown that the causal agent of this tick-borne fever is perfectly distinct from the virus of louping ill (see Gordon *et al*, 1932 *a*, *b*, MacLeod and Gordon, 1932, 1933)

Immunity reactions and prevention

Many animals on an infected farm are immune to injections of virus, presumably owing to a previous mild or abortive attack giving rise to a state of active immunity

Subcutaneous inoculations of formolized sheep cord and brain produce antibodies capable of neutralizing virus introduced into the general circulation, although intracerebral injections are still capable of inciting infection Such vaccines can be used in prophylaxis of the disease, the vaccine has been successfully used on a large scale by Gordon and his associates (see Edward, 1947 *b*) Edward (1947 *b*) reports good results in protecting sheep by the use of formolized vaccine containing sheep brain, cord, and spleen The challenge virus was given subcutaneously, and 3 days later starch was given cerebrally It was argued that in immune animals virus would not proliferate in the blood, and so the cerebral starch would not localize virus in the brain

Animals that have withstood intranasal, intradermal, or subcutaneous injections of living virus resist intracerebral injection Unfortunately this method of vaccination appears to be rather dangerous

In an article (*Bulletin*, 1938), which should be consulted by those interested in louping ill, practical methods of dealing with ticks by dipping and other measures are discussed

LOUPING ILL IN MAN

Although louping ill has been recognized in sheep for over a century, there has never been any suggestion of transmission to human beings taking place under natural conditions Thus there have been no reports of unusual nervous symptoms in shepherds, farmers, veterinary surgeons, or others coming in contact with in-

CHAPTER XCIX

LOUPING ILL[1]

This disease of sheep, occurring in Scotland and the north of England, has been known for over a century, and during the past 50 years numerous attempts have been made to define its causal agent (see Pool, Brownlee, and Wilson, 1930, Pool, 1931 *a*, *b*; Pool, 1933–4) A variety of bacteria, toxemias, and other causes has been blamed, but no real progress resulted until Pool, Brownlee, and Wilson (1930) succeeded in infecting both pigs and sheep experimentally, by intracerebral injections of sheep brain and cord.

Mackie (see Alston and Gibson, 1931) was the first to demonstrate that the etiological agent was a filtrable virus, by preparing infective Berkefeld filtrates of sheep brain. Under his direction Alston and Gibson (1931) succeeded in transmitting louping ill to mice by intracerebral injection. The discovery of the susceptibility of mice enormously facilitated the subsequent investigations into the natural disease. Greig *et al.* (1931) demonstrated that Berkefeld and Chamberland filtrates were capable of infecting sheep.

The bulk of the work on louping ill in sheep has been carried out in Scotland at the Moredun Institute, Midlothian

Louping Ill in Sheep

The following account is largely summarized from the publications of the Moredun investigators (Pool, Brownlee, and Wilson, 1930, Greig *et al*, 1931; Pool, 1931 *a*, *b*; Brownlee and Wilson, 1932, Gordon *et al*, 1932 *a*, *b*, MacLeod and Gordon, 1932, Gordon, 1933–4, 1934; Pool, 1933–4)

Features of the disease

One of the most characteristic features is a diphasic febrile reaction During the prodromal period the animal is indisposed, the temperature raised, and the virus can be demonstrated, by animal injection, in the blood Many mild and abortive cases may not show any further evidence of infection with louping ill than this preliminary pyrexia Such cases can only be diagnosed with certainty by injection of mice with citrated blood In severer cases, however, symptoms of invasion of the central nervous system subsequently appear, along with a second attack of pyrexia During this attack, moreover, no virus is demonstrable in the blood It has been shown (*vide infra*) that another disease of sheep, tick-borne fever, is an important factor predisposing to involvement of the central nervous system

The commonest nervous symptoms are champing of the jaws, tremors and "tremblings," paresis of one or more limbs, cerebellar ataxia, and leaping movements into the air. The case mortality rate is high

Pathology.

The pathological features of the naturally occurring disease have been well described by Brownlee and Wilson (1932) and are those of an acute encephalomyelitis

Histologically, the cerebral meninges show a diffuse congestion and infiltration with round cells and occasional polymorphs The cerebral cortex shows the following changes in varying degree perivascular cuffing, degenerative changes in the nerve cells, and diffuse infiltration with polymorphs

The main changes are, however, to be found in the cerebellum The meninges are congested and infiltrated with round cells The substance of the cerebellum shows diffuse

[1] Although this disease is primarily one of animals, and only rarely occurs in man, we have discussed it fully in view of the possibility that the viruses of louping ill and Australian X disease are identical, or else closely related (see also Ch C)

inasmuch as no virus was isolated from the brain by monkey or mouse inoculation, and as no serum was available, we do not consider the evidence sufficient to justify any conclusions as to whether this case was one of louping ill or not. If it were a case, then some feasible explanation of how the child came to be infected with the virus should be forthcoming.

CHARACTERISTICS OF THE LOUPING ILL VIRUS

Animal Experiments

Sheep.

The infection has been transmitted to sheep (e g, Pool, Brownlee, and Wilson, 1930, Greig *et al*, 1931; Brownlee and Wilson, 1932, Lépine, 1931 *a*, Edward, 1947 *b*).

Following intracrebral injection a thermal reaction occurs (above 106° F), reaching its acme about the 4th to 5th day and then subsiding In certain cases the sheep then becomes dull and shows incoordination, tremors, salivation, and champing of the jaws A day or two later it becomes paralyzed and falls to the ground, eventually dying in coma

Histologically, the cerebrum shows a well marked meningeal reaction, and in the cortex perivascular cuffing and some diffuse cellular infiltration The cerebellum shows a varying destruction of the Purkinje cells, with neuronophagia and accompanying microglial proliferation Perivascular cuffing may be present in addition, in the hippocampus, medulla, and cord The Golgi apparatus of the nerve cell undergoes changes consequent on invasion by the virus There is hypertrophy, a reduction in the number of filamentous elements, and a reduction in the amount of Golgi substance in the cell processes, fragmentation ensues (Gresson and Zlotnik, 1947).

The sheep can also be infected by the intraspinal, intra-ocular, ocular, intrasciatic, intravenous, and subcutaneous routes Edward (1947 *b*) could only produce encephalitis in about half the sheep inoculated subcutaneously In some animals inoculated in June and July no encephalitis occurred, pointing to the importance of seasonal and other factors determining invasion of the brain after local growth of virus Encephalitis occurred with greater regularity if starch was injected cerebrally

The virus is widely distributed in the brain, cord, sciatic nerve, blood (from 3 to 7 days after inoculation), spleen, voluntary muscle, and popliteal and mesenteric lymph glands of infected sheep

Pigs.

The virus can also be transmitted to the pig, but serial passage is not possible (Pool, Brownlee, and Wilson, 1930, Greig *et al*, 1931, Brownlee and Wilson, 1932) Typical louping ill develops after 5 to 6 days

Histologically, the reaction is of a different order from that found in the sheep and monkey, being predominantly interstitial The meningeal reaction is pronounced, and there is much perivascular cuffing The Purkinje cell layer is widely infiltrated, but there is relatively little cell destruction

Mice

Following the discovery made by Alston and Gibson (1931) the high susceptibility of mice has been abundantly confirmed (Greig *et al*, 1931, Lépine, 1931 *c*, Czardowska-Gladney and Hurst, 1931, Hurst, 1931, Brownlee and Wilson, 1932, Webster and Fite, 1932–3, Fite and Webster, 1933–4, Haber, 1939 *a*, *b*, *c*, Edward, 1947 *b*) Strains specially susceptible or resistant can be bred (Webster and Fite, 1932–3, Casals and Schneider, 1943).

An incubation period of 5 to 9 days follows intracerebral injection. During the last 12 to 24 hours prodromata may appear, such as irritability and hyperesthesia, or sometimes immobility. The symptoms of the established disease are as follows

fected sheep. There does not appear to be any method whereby the virus can become transmitted from a sheep suffering from louping ill to man.

Of great interest, however, are certain records of cases of louping ill infection occurring in laboratory workers. Rivers and Schwentker (1932–3, 1934) were the first to draw attention to this hitherto unreported human disease. They described 4 cases, each person having worked, at the time of being taken ill, with louping ill [illegible] at Britain. Subsequently Wiebel [illegible] worker, this time in Germany [illegible]

Presumably, infection was massive, with infective animal tissue, the virus probably entering via the respiratory passages.

Clinical Features

Prodromal period. Two of Rivers's and Schwentker's cases had a preliminary illness lasting approximately a week. The patients were febrile, suffered from headache, malaise, and backache, and were evidently prostrated. The cases then improved and kept well for about another week when the symptoms of the disease proper appeared.

Course of the disease. The following symptoms arose either *de novo* or after prodromata Nervous manifestations included headache, diplopia, drowsiness and mental confusion, photophobia, stiffness of the neck, vomiting, blurring of the optic disks, and weakening of the deep reflexes. Cerebellar ataxia was noted only in Wiebel's case A retrobulbar neuritis developed in one of Rivers's cases The cerebrospinal fluid was frequently blood-tinged, apparently unassociated with trauma, the pressure was raised, the cell count varied up to 60 cells per c mm, mainly mononuclears, and there was often an increase of globulin General pyrexial disturbances were always present and the blood showed a polynucleosis up to 17,000 per c mm Two of Rivers's cases were originally diagnosed as epidemic encephalitis, one as influenza, and the 4th as tuberculous meningitis Rivers and Schwentker failed to find any virus in the blood and cerebrospinal fluid of 2 cases examined. Wiebel, however, isolated the virus from the serum of his case.

Immunity Reactions

On recovery all the 5 cases described showed virus neutralizing serum antibodies This fact clinched the originally somewhat tentative diagnosis of human infection with louping ill virus.

Rivers and Schwentker found virus neutralizing bodies in the serum of another laboratory worker and in one other person, out of almost 60 tests on various human sera. Neither the laboratory worker nor the other person (a sufferer from obesity) had had any recent manifestations of illness However, whereas the first person had been in contact with the virus, the second had never had the remotest contact therewith, as far as could be ascertained Complement fixing and virus neutralizing antibodies probably persist for at least 10 years (Casals and Webster, 1944).

Johnston and Goodpasture's (1936) Case

These workers recorded the case of a Negro child who developed a fatal nervous disease some few days after a burn The child died in coma after showing convulsions The whole illness was of very short duration Postmortem changes were restricted to the brain and were those of an acute, diffuse, nonsuppurative encephalitis. The cerebrum, basal ganglia, pons, medulla, and cerebellum showed a perivascular lymphocytic infiltration The important change, however, concerned the cerebellum where there was gross loss of Purkinje cells, with necrosis and neuronophagia. On the strength of the similarity of the child's lesions to those of experimental louping ill of monkeys, Johnston and Goodpasture suggested they were dealing with a virus producing lesions resembling those of louping ill However,

ing degrees of perivascular cuffing, nerve cell destruction, and gliosis. Generally, if the lesions are advanced in the cortex they are mild in the stem and cord, and vice versa.

The infection can also be transmitted intranasally (under light etherization) as Galloway and Perdrau (1935) showed using *Macaca mulatta*. After an incubation period of approximately a fortnight symptoms appear, exactly as already described. The path followed to the central nervous system is a steadily progressive one. After 5 days no virus can be demonstrated in the brain, but it appears after 8 days in the olfactory lobe. After 12 days it can be demonstrated in all parts of the brain and cervical cord, while by the 16th day it has spread even to the peripheral nerves.

No infection results after intravenous inoculation unless the brain has been previously traumatized (Galloway and Perdrau, 1935).

Following intracerebral inoculation the virus appears in the blood after 2 to 4 days, but disappears shortly before death The virus can be demonstrated in the cerebrospinal fluid after intranasal inoculation, but only rarely in the blood or elsewhere.

Other animals.

Field voles These animals resemble mice in their susceptibility and can be infected intracerebrally, and by intraperitoneal injection if the virus is localized in the brain by an injection of starch (Findlay and Elton, 1933). Histologically, the lesions resemble those described in mice by Hurst (*vide supra*). It should be remembered that voles live in pastures affected by louping ill, and may conceivably play some part in the spread of the disease.

Rats. Burnet (1936 *b*) has shown that rats acquire an inapparent (subclinical) infection after intranasal as well as after intracerebral inoculation (see also Greig *et* ... or 8th da[illegible] ... These an ... spread to

Other experiments.

Experiments have been conducted on the simultaneous infection of mice with louping ill and rabies, and louping ill and LGI viruses (Levaditi, 1942 *a*, *b*).

Morphology

The virus measures from 15 mμ to 20 mμ as estimated by filtration through collodion membranes (Elford and Galloway, 1933), and 27 mμ to 22 mμ as estimated by the inverted capillary tube technique (Tang, Elford, and Galloway, 1937). The eosinophilic inclusions of Hurst (1931) have already been described as occurring in mouse brain Findlay (1932) found similar bodies in monkey tissue, which, as they [illegible]

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Cultivation

Rivers and Ward (1932–3), using chick embryo, monkey serum, and Tyrode's solution, obtained definite evidence of virus multiplication in tissue cultures of the Li and Rivers type (see also Wilson, 1945).

Burnet (1936 *a*, *b*) found the virus to be propagated readily in the developing hen egg, producing numerous foci of opacity (see also Burnet, Keogh, and Lush, 1937)

The embryo is frequently killed by the virus and appears jaundiced, but some-

tremblings, tremors, spasmodic contractions of the head and thoracic muscles, clonic or tonic convulsions, and incoordination. Shortly before death, paresis of one or more limbs occurs and the animal is usually dead by the 8th to 10th day after injection.

Histologically, brains of mice dying from encephalitis show marked perivascular and diffuse lymphocytic infiltrations in the basal nuclei and cerebral cortex. In the brain stem and spinal cord acute nerve cell destruction is the most noticeable feature. There is widespread neuronophagia in the cord and the appearances closely resemble those of poliomyelitis. The cerebellum may be severely damaged with nerve cell destruction or may escape in a remarkable way.

Inclusion bodies have been described by Hurst (1931); they occur in the neurons of the brain stem and cord and less commonly in the Purkinje cells and cells of the basal nuclei. They are found in the cytoplasm, as single or multiple, eosinophilic, round or oval bodies of 1 to 8 μ in diameter.

Mice can also be infected intranasally (Webster and Fite, 1932–3; Webster, 1933, Fite and Webster, 1933–4). Following such instillation virus is present in the blood from the 2nd to the 6th days, when the animals become ill (with features already described) and the virus tends to disappear. As a rule, virus is not generally present in the brain until the 3rd day, nor are histological lesions noted until the 5th day. Rake (1937) has shown that the virus has reached the forebrain 24 hours after nasal instillation.

Alston and Gibson (1931) noted contact infection in some of their cages, but this method of spread does not appear to have been recorded by other workers. The virus can be transmitted by intrasciatic and intra-ocular injections, encephalitis developing (Lépine, 1931 c).

After 40 mouse passages the virus still retained its infectivity for sheep (Elford and Galloway, 1933) The virus is widely distributed in the internal organs of mice dead of louping ill infection.

Burnet and Lush (1938) have carried out an important study on the pathogenesis of infection following injection by routes other than the intracerebral As indicators of the presence of virus in various tissues they used the chorio-allantois in lieu of animals

After intraperitoneal injection they found that the virus usually enters the central nervous system by the olfactory mucosa, and spreads by the olfactory bulbs They showed that trauma to the cerebrum permits virus to enter at the site of damage

Following intranasal injection the virus soon enters the olfactory bulbs, titrations showed that the virus proliferates there to a considerable extent before it can be detected in other parts of the brain Later, virus disseminates widely through the nervous system and can be found even in the sciatic nerve

Monkeys.

The infection has been transmitted to monkeys by Hurst (1931), Findlay (1932), and Galloway and Perdrau (1935).

Some 4 to 5 days after intracerebral inoculation of virus the animal becomes less active, and shows muscular weakness and tremors, while nystagmus may occur With the progress of the infection evidences of cerebellar ataxia become noticeable, the animal is clumsy, unsteady, and bumps into obstacles Eventually the animal collapses and lies on its side Despite passing through such a severe infection recovery may occur. Histologically, the cerebellar lesions are the most noticeable There is widespread destruction and sometimes total disappearance of the Purkinje cells, but little reactionary gliosis Inclusions similar to those described by Hurst (1931) in the mouse have been described in the monkey by Findlay (1932) They occur most frequently in the neurons of the pons and midbrain and less commonly in the spinal cord and Purkinje cells The cortex, brain stem, and cord show vary-

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Guinea-pigs and *rabbits* cannot be infected (Casals and Webster, 1944).

Other experiments.

Experiments have been conducted on the simultaneous infection of mice with louping ill and rabies, and louping ill and LGI viruses (Levaditi, 1942 *a*, *b*).

Morphology

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The virus passes Berkefeld V, N, and W, Chamberland L_2 and L_3 filters (Alston and Gibson, 1931, Greig *et al*, 1931, Lépine, 1931 *b*) The virus is negatively charged at pH 7 3 (Lépine, 1931 *b*).

Cultivation

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Valley fever and louping ill viruses The viruses of louping ill and Colorado fever are unrelated (Koprowski and Cox, 1947)

The relationship to Australian X disease is discussed in Ch C.

Although differing somewhat in biological properties, louping ill virus is genically closely related to that of Russian spring-summer encephalitis, as sh by complement fixation, neutralization, and intraperitoneal cross resistance tes mice (Casals, 1944; Casals and Webster, 1943, 1944, see also Silber and Solo 1946). Russian workers claim to have isolated true louping ill virus from *Ix ricinus* ticks in White Russia (Silber and Shubladze, 1945).

There is some disagreement as to the relationship of louping ill and Ru viruses to those of Japanese and St. Louis encephalitis (see Ch. XCVIII).

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times edema is present instead The liver is either deep greenish brown without necrosis, or else of normal color with extensive necrotic patches. The virus is present in the embryo blood and washed red cells from the 2nd to the 6th days after inoculation. The virus can be liberated from the red cells by treatment with distilled water. In the egg, therefore, the virus is strongly viscerotropic.

Edward (1947 *a*) has propagated the virus by inoculation in the yolk sac or embryo. The yolk sac route can be used to titrate virus suspensions or to test for virus neutralizing antibodies. The egg virus appears to [illegible] for the sheep when [illegible] in severe wintry [illegible] this was attributed to incr[illegible] the CNS, for later passage material inoculated in the summer was relatively innocuous (1947 *b*).

Serum neutralization tests can also be carried out using the pock counting method.

Reaction to Physical and Chemical Agents

The virus is heat sensitive, being destroyed at 58° C in 10 minutes, 60° C. in 2 to 5 minutes, and 80° C. in 30 seconds (Lépine, 1931 *b*).

Frozen and kept in a Petri dish in the refrigerator, the virus does not survive much over 11 to 14 days (Lépine, 1931 *b*)

The virus can be readily preserved for storage by pulping, drying *in vacuo* over sulfuric acid, and storing in the refrigerator for about 14 days. The powder is then dry and can be stored in a desiccator (Greig *et al*, 1931).

The virus can survive in glycerol for approximately 4 to 6 months (Lépine, 1931 *b*, Czardowska-Gladney and Hurst, 1931). The virus in tissue-free suspensions is rapidly inactivated by methylene blue in the presence of light (Perdrau and Todd, 1936). The virus is inactivated by bile salts, the virus-inactivating agent, saponin, sodium desoxycholate, and sodium lauryl sulfate (Burnet, Lush, and Jackson, 1939; Smith, 1939, Burnet and Lush, 1940).

Immunity Reactions in Experimental Animals

On recovery from infection, animals are resistant to reinoculation (Greig *et al*, 1931, Lépine, 1931 *c*, Findlay, 1932, Schwentker, Rivers, and Finkelstein, 1933, Galloway and Perdrau, 1935, Burnet, 1936 *b*).

Edward (1947 *b*) vaccinated mice with formolized virus in mouse brain, so that they resisted peritoneal or nasal challenge

Virus neutralizing antibodies develop in animals on recovery from infection, or after inoculations of virus by various routes in sheep, rats, monkeys, and mice Antibodies can be titrated by pock counting (Burnet, 1936 *a*, *b*), in the yolk sac (Edward, 1947 *a*), or by mouse inoculation Mixtures apparently neutral can be reactivated, and the reaction is reversible (Goyal, 1935, Burnet, Keogh, and Lush, 1937)

Complement fixation can be demonstrated between various types of brain extract and immune sera (Casals and Palacios, 1941 *a*, *b*, Casals, 1945) Casals' latest (1947) technique is described in Ch XI

Relationship to Other Virus Diseases

Although there is a certain superficial similarity in the histological appearances of poliomyelitis and louping ill, especially as regards nerve destruction and perivascular cuffing in the cord, the viruses are not closely related (Schwentker, Rivers and Finkelstein, 1933). These workers showed, first that monkeys refractory to louping ill were susceptible to poliomyelitis and vice versa, and secondly that serum neutralization tests elicited no cross reactions between the two viruses.

Findlay (1932) has shown that no cross immunity reactions occur between Rift

CHAPTER C

AUSTRALIAN X DISEASE

CLINICAL AND PATHOLOGICAL FEATURES

THE clinical features were described by a number of authors (e g, Litchfield, 1917, Cleland and Bradley, 1917; Cleland, Bradley, and Buckley, 1917, Breinl, 1917, Cleland and Campbell, 1919 *a*, *b*, 1919–20 *b*, 1920, Kneebone and Cleland, 1926).

Incubation period As in so many similar disorders it proved difficult to estimate the incubation period, but it was probably 5 to 12 days

Prodromal symptoms. These included headache, pains in the body, mental irritability or confusion, drowsiness, and weakness of the limbs.

Course. The disease was ushered in acutely (especially in children) by vomiting, fever, and convulsions. In adults the following initial symptoms were commoner. headache, neck pains, confusion, drowsiness or lethargy, muscular weakness, and incoordination

The temperature, at first 101° to 102° F., rose to 104° to 105° F. with the progress of the infection. Convulsive movements were common, especially in chil- [illegible] these movements often took [illegible] s tremors and twitchings of [illegible] were observed, and muscular rigidity was frequently present. Paralyses of eye muscles, face, or limbs were exceedingly rare The cerebrospinal fluid usually showed a well-marked pleocytosis.

The duration of the illness was usually from 10 to 12 days, after which the patient recovered rapidly. Cases proving fatal usually did so from 4 to 6 days after the onset, but in certain fulminant cases death occurred in 24 hours. The fatality rate was 70 per cent

The characteristic postmortem findings have been described by a number of authors (Breinl, 1917, Cleland, 1918–19, Cleland and Campbell, 1919 *a*, 1919–20 *b*, 1920), and have been reexamined by Perdrau (1936).

The changes were practically restricted to the nervous system, being evident in the cerebral cortex, basal nuclei, dentate nucleus of the cerebellum, the dorsal parts of the pons and medulla, and the cornua of the cord They were more pronounced in the encephalon than in the cord.

The vessels and plexuses were engorged and there were numerous petechial hemorrhages in the meninges and [illegible] in the meninges, perivascularly, [illegible]

EPIDEMIOLOGY

The epidemiological features have been fully described by Cleland and Campbell (1919 *a*, 1919–20 *a*,*b*, 1920)

In the 1917 and 1918 epidemics the country towns of New South Wales, Queensland, and Victoria were mainly involved An outbreak occurred in *1925 in* the Broken Hill district (Kneebone and Cleland, 1926).

The usual time of year for these outbreaks to occur was between January and April, with the maximum number of cases in February and March, that is to say, in the hot, dry weather

Nearly 50 per cent. of the cases occurred in individuals under 5 years of age. Males were proportionately more susceptible than females (85 56).

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Course The disease was ushered in acutely (especially in children) by vomiting, fever, and convulsions In adults the following initial symptoms were commoner headache, neck pains, confusion, drowsiness or lethargy, muscular weakness, and incoordination.

The temperature, at first 101° to 102° F., rose to 104° to 105° F with the progress of the infection. Convulsive movements were common, especially in children With the deepening of lethargy and drowsiness these movements often took the form of thrashing of the bedclothes In other cases tremors and twitchings of face and limbs, teeth grinding, eye-blinking, and hiccup were observed, and muscular rigidity was frequently present Paralyses of eye muscles, face, or limbs were exceedingly rare The cerebrospinal fluid usually showed a well-marked pleocytosis

The duration of the illness was usually from 10 to 12 days, after which the patient recovered rapidly. Cases proving fatal usually did so from 4 to 6 days after the onset, but in certain fulminant cases death occurred in 24 hours The fatality rate was 70 per cent

The characteristic postmortem findings have been described by a number of authors (Breinl, 1917, Cleland, 1918–19, Cleland and Campbell, 1919 *a*, 1919–20 *b*, 1920), and have been reexamined by Perdrau (1936)

The changes were practically restricted to the nervous system, being evident in the cerebral cortex, basal nuclei, dentate nucleus of the cerebellum, the dorsal parts of the pons and medulla, and the cornua of the cord They were more pronounced in the encephalon than in the cord

The vessels and plexuses were engorged and there were numerous petechial
hemorrhages in the [illegible]

EPIDEMIOLOGY

The epidemiological features have been fully described by Cleland and Campbell (1919 *a*, 1919–20 *a*,*b*, 1920)

In the 1917 and 1918 epidemics the country towns of New South Wales, Queensland, and Victoria were mainly involved. An outbreak occurred in 1925 in the Broken Hill district (Kneebone and Cleland, 1925).

The usual time of year for these outbreaks to occur was between January and April, with the maximum number of cases in February and March, that is in the hot, dry weather.

Nearly 50 per cent of the cases occurred in individuals under 5 years of age. Males were proportionately more susceptible than females (55–56).

Some evidence of contact infection was obtained, but only rarely. An investigation was made into the possible rôle of invertebrate hosts, but no evidence was found to incriminate ticks, bugs, flies, lice, or mosquitoes Modern Australian opinion, however, would appear to hold to the possibility that the disease might have been spread by flies (see Burnet, 1934).

ISOLATION OF VIRUS FROM CASES OF X DISEASE

The main work was carried out by Cleland and his associates (see Cleland and Campbell, 1919 *a*, *b*, 1919–20 *a*, *b*, *c*, Cleland, 1918–19, Kneebone and Cleland, 1926). Breinl (1917, 1918) also isolated a strain of virus from cases of the disease by intracerebral injection of a cercopithecus monkey. These workers isolated strains in 1917, 1918, and 1925.

Animal Experiments

Monkeys. Rhesus or cercopithecus monkeys were infected by intracerebral injection. There was an incubation period of some 5 to 23 days before the animal sickened, showing dullness, lethargy, muscular weakness, incoordination, tremors, and rigidity. In 70 per cent. of animals pareses were noted. Finally convulsions might occur, the animal becoming prostrate and dying in from 2½ to 6 days

Histologically the lesions resembled those of human beings the meninges were congested and showed round cell infiltration, the brain substance was congested, the vessels showed perivascular cuffing, and there were foci of round cell infiltration There was almost total loss of Purkinje cells in the cerebellum (Perdrau, 1936).

Sheep The animals sickened after an incubation period of from 3 to 12 days The head drooped, and there were tremors, convulsions, teeth grinding, jaw champing, drowsiness, and a staggering gait.

Histologically there were perivascular cuffing and other changes as described in human cases. Destruction of Purkinje cells was not noted

Horse and calf Both these animals were infected and developed, after 5 to 9 days' incubation, convulsions, staggering, and twitchings

Insusceptible animals The infection could not be transmitted to the following animals dogs, kittens, rabbits, guinea-pigs, or fowls.

Some Properties of the Virus

The virus was not conclusively shown to pass a Berkefeld filter.

It did not survive in glycerol for over 2 months. Neutralizing antibodies developed in the sera of convalescent sheep (Cleland and Campbell, 1919–20 *b*, *c*)

RELATIONSHIP TO OTHER INFECTIONS

At the outset it was suggested that the X disease was an exalted form of poliomyelitis (Flexner, 1923) The histological pictures were, however, somewhat different, and the ease of transmission of X disease virus to horses and sheep definitely showed it to be a distinct entity. In addition, the sera of poliomyelitis convalescents proved incapable of neutralizing the virus (Cleland and Campbell, 1919–20 *c*)

At the time of the isolation of Cleland's virus the etiological agent of louping ill was unknown Inasmuch as Cleland's virus is no longer available, arguments have to be based largely on facts previously ascertained Reviews have been published by Burnet (1934) and Perdrau (1936)

No cases of louping ill in man have proved fatal (see Ch XCIX) It has not, therefore, been possible to compare sections of human material from the two diseases, one with another The brains of fatal human cases of X disease showed destruction of the Purkinje cells similar to that observed in the brains of monkeys infected with louping ill. It should be mentioned at this point that cases of widespread loss of Purkinje cells, unassociated with other lesions, have been recorded in

man, quite unconnected with louping ill or X disease (Parker and Kernohan, 1933, Greenfield, 1934).

The characteristic feature of louping ill in animals is the great destruction of the Purkinje cells that occurs, and monkeys infected with X disease showed much the same type of change. Further, the symptoms in monkeys injected are similar, although X disease produced more pareses.

To set on the other side of this question are the following facts: The symptoms in injected monkeys are not exactly the same. Neither is *Ixodes ricinus* known in Australia, nor are other species of tick common. The common ectoparasite of Australian sheep is *Melophagus ovinus*, but this remains all its life on one host (Burnet, 1934). Louping ill has never been reported in Australia, and certainly at the time no unknown disease was present in sheep in the infected localities.

It will not be possible to elucidate the matter further unless there are any more outbreaks, but the two agents are probably closely related.

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CHAPTER CI

ASCENDING MYELITIS DUE TO B VIRUS

CLINICAL AND PATHOLOGICAL FEATURES

A LABORATORY worker developed a spreading infection after a bite on the hand from a monkey. He developed ascending myelitis, and died after 2 weeks (Gay and Holden, *1932–3, 1933 a, b, Sabin and Wright, 1934*)

The bite wound itself was healing well but the regional glands were hemorrhagic. Naked-eye, the brain appeared healthy, and the cord only slightly edematous The spleen was enlarged and of soft consistency. Microscopically, the change in the cord was a transverse myelitis of the mid-cervical and upper dorsal regions The medulla showed the severest lesions of any part of the central nervous system, where there was an extremely severe infiltration of polymorphs and lymphocytes, most pronounced near the floor of the 4th ventricle. Marked mononuclear perivascular cuffing was present in the pons, basal ganglia, internal capsule, and uncinate gyrus. No demyelination was noted in any part of the central nervous system. The spleen was hyperemic and showed necrotic foci, similar foci were also present in the adrenals and lymph glands Two different strains of virus were isolated.

Gay and Holden (1932–3, 1933 *a, b*) isolated W virus, and Sabin and Wright (1934) isolated B virus Sabin and Wright's work was expanded by Sabin (1934 *a, b, c*), Sabin and Hurst (1935), and Haber (1935)

CHARACTERISTICS OF B VIRUS

Infection in Rabbits

Rabbits were found to be infected readily (Sabin and Wright, 1934; Sabin, 1934 *a, b, c*, Haber, 1935). Rabbits are susceptible to intracerebral injection and die somewhat suddenly on about the 5th day. Before death, raised temperature, salivation, and convulsions may all be noted, but no paresis of the extremities

After intradermal injection, preferably in the abdomen, the virus shows a most pronounced neurotropism About 24 hours after injection erythematous papules are evident locally, and soon become hemorrhagic and necrotic centrally About the 6th day the temperature rises with the appearance of paresis of one or both posterior extremities Within another day the paralysis has spread to involve the fore limbs Seven to 12 days after injection, urinary retention, salivation, and convulsive movements of the head precede death

A fatal paralysis follows intraperitoneal, intravenous, intramuscular, intratesticular and subcutaneous injections

After corneal scarification a fatal keratitis develops, the animal can also be inoculated intranasally (Haber, 1935, Sabin, 1934 *b*)

The virus must spread to the central nervous system by the nerve trunks following peripheral injection, as after intracutaneous injection it can be found in the cord while still absent from the brain.

After intracerebral injection a comparatively mild meningitis develops, with scanty fibrinous exudate over the cerebrum and cerebellum (Sabin and Hurst, 1935).

At the *site of injection* in the cortex there is some slight necrosis of nerve cells [illegible] inclusions are found in the glial cells The [illegible] l and subependymal glia, and in the cortical [illegible] cortical vessels is noted

After intracutaneous [illegible] *hemorrhagic necrotic lesion* forms locally

There is extensive necrosis of the cutis and subcutaneous tissues with infiltration by polymorphs and mononuclears. At the periphery of the lesion typical inclusions are to be found. Spread of the infective process occurs and degeneration and polymorph infiltration can be demonstrated in the appropriate spinal ganglia. Next, the corresponding segments of the cord are involved by severe degenerative and inflammatory reaction, converting the tissue into a diffluent mass Inclusion bodies are found in the nerve cells and glia before the appearance of these changes. Rabbits injected intracutaneously, as well as by other peripheral routes, show lesions in the internal organs

After intramuscular injection a severe necrosis of muscle cells, vessels, and nerves occurs The sciatic nerve shows an interstitial neuritis while inclusions are present in the connective tissue cells and the sheath of Schwann.

After intravenous injection necrotic foci are present in the adrenal and less commonly in the liver and spleen

After intraperitoneal injection foci of necrosis can be found in the adrenal, liver, spleen, and ovary.

Monkeys.

The virus is pathogenic for *M rhesus* and cebus monkeys Callithrix monkeys can be infected also, but not cynomolgus (Haber, 1935)

After intracerebral inoculation, paresis, rigidity, and incoordination develop and death frequently follows

After intravenous injection an eruption appears on the forehead, conjunctivae, face, tongue, and buccal mucosa

Intracutaneous injection is followed by an acute hemorrhagic and necrotic lesion, again resembling a vaccinial pock

Intraperitoneal injection results in the development of peritonitis.

Pathology of infected monkeys (see Sabin and Hurst, 1935)

After intracerebral injection, as contrasted with rabbits, a gross meningitis develops, the pia over the cortex, stem, and cerebellum being infiltrated with fibrinous exudate in which are polymorphs and mononuclears Inclusions are to be found in the meningeal vessel cells as a prelude to necrosis The adventitial and muscular coats are frequently severely affected by this degenerative process The site of inoculation is involved in a hemorrhagic [illegible]

[illegible]

[illegible] and loss of Nissl bodies, some infiltration with polymorphs and proliferation of glia are usually present After peripheral injection in monkeys the nervous lesions are very mild, but focal necroses may be found in the liver, spleen, adrenal, and ovary

Other animals.

Guinea-pigs [illegible] bral injection fatal [illegible] do not occur, but [illegible]

Mice develc [illegible]

Certain Other Properties of B Virus

Elementary bodies have not been described, although inclusion bodies have been found (Sabin and Hurst, 1935). These bodies are intranuclear, and occur in cells in the central nervous system and internal organs They resemble closely the inclusions of herpes, pseudorabies, virus III, and salivary gland virus of guinea-pigs It has been proved possible to demonstrate the inclusions of B virus in superinfected Shope's papillomatous tumors in rabbits (Syverton and Berry, 1947*a*), the inclu-

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At the site of injection in the cortex there is some slight necrosis of nerve cells and numerous eosinophilic intranuclear inclusions are found in the glial cells The inclusions are widespread in the subpial and subependymal glia, and in the cortical nerve cells. Mononuclear cuffing of the cortical vessels is noted

After intracutaneous injection a hemorrhagic necrotic lesion forms locally.

paresis of the lower extremities Intranuclear inclusions are readily found, and in Ammon's horn involve nearly every cell After intradermal injection, the results are similar to those obtained with B virus, an ascending paresis following a local hemorrhagic and necrotic lesion

Rhesus monkeys cannot, but cebus monkeys can, be infected on intracerebral injection, the animals dying in 7 days after showing spastic paresis, lethargy, and convulsions.

Gay and Holden regarded the properties of W virus and of herpes as identical However, the results on intradermal injection of rabbits with W virus resemble exactly those of B virus, and are more pronounced than in the case of herpes

To confirm their view, these authors undertook some cross immunity tests with Perdrau's E L.1 strain of herpes Among other experiments they found that antiherpetic rabbit serum modified but did not completely neutralize W virus. They came to the final conclusion that W virus was a strain of herpes

CONCLUSIONS

It appears that B and W viruses are identical, and this virus, although closely related to herpes, is probably distinct.

In our opinion, it is most probable that the infection was transmitted by the monkey, even though Haber (1935) has not been able to demonstrate the virus in a number of specimens of saliva The virus has unusual properties, differing from the common type of herpetic strains, and in all probability may be normally carried by monkeys in captivity, especially as Sabin, and Burnet, Lush, and Jackson (1939 *b*) found antibodies in monkey sera

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sions of vaccinia and B virus have been demonstrated in the cytoplasm and nucleus respectively of individual cells of the rabbit cornea (1947 *b*).

As regards filtrability, the virus passes through a Berkefeld V and N, a Chamberland L_3, and a single disk Seitz (Sabin and Wright, 1934; Sabin, 1934 *b, c*).

It is deposited in 3¼ hours at 14,000 r.p.m. (Sabin, 1934 *b*)

The virus resists glycerol for at least several months. It is sensitive to the virus-inactivating agent in normal human nasal secretion which acts powerfully on influenza virus, it is also inactivated by saponin, sodium desoxycholate, and sodium lauryl sulfate (Burnet, Lush, and Jackson, 1939 *c*; Burnet and Lush, 1940).

Shocking doses of metrazol or insulin did not influence the course of infection in adult mice (Holden, 1940). B virus produces pocks on the chorio-allantois (Burnet, Lush, and Jackson, 1939 *a, b, c*).

Relationship to Herpes

Resemblances can be recapitulated as follows. (*a*) development of encephalitis in rabbits after intracerebral or corneal inoculation of virus, the 2 histological pictures being very similar, (*b*) development of adrenal necrosis after intravenous injection of rabbits, (*c*) production of myelitis after intradermal injection of rabbits (note, however, this is much commoner with B than herpes); (*d*) similar intranuclear inclusions, (*e*) similar effect on cebus monkeys

There are, however, certain differences Thus, after intratesticular injection, myelitis develops with B, but encephalitis with herpes. Rhesus monkeys are susceptible to B only. After intravenous injection necrotic lesions of the spleen and liver only occur with B It appears to us that these differences, although only in degree, are sufficient justification for regarding B virus as a distinct entity. This claim is further strengthened by the following cross immunity reactions carried out by Sabin. Herpes-immune rabbits generally succumb from an intradermal injection of B virus, but occasionally a small dose may prove noninfective In support of this it was found that herpes-immune sera (guinea-pig) are usually quite inactive against B virus but rarely may neutralize a minimal dose, vice versa, immune B sera may neutralize a minimal dose of herpes

This problem has been investigated by Burnet, Lush, and Jackson (1939 *b*), by the chorio-allantoic technique They found antibody to B virus in the sera of Australian monkeys, and showed that these sera inactivated herpes to a comparable titer. The inoculation of monkeys with B resulted in the appearance of antibodies which were considerably more active against herpes than B virus They concluded that, as demonstrated by the egg technique, B is a more complete antigen than herpes virus.

Relationship to Pseudorabies and Other Viruses

Cross immunity reactions (Sabin, 1934 *c*) have been carried out Briefly, anti-B sera may neutralize minimal amounts of pseudorabies, guinea-pigs convalescent from B infection may resist minimal doses of pseudorabies, and one monkey solidly immune to B was found to be resistant to pseudorabies It appears, then, that the relationship to pseudorabies is of the same order as that to herpes B virus is allied but distinct.

Cross immunity tests with neutralizing antisera or immune animals failed to show any relationship to equine encephalomyelitis, virus III of rabbits, or vaccinia (Sabin, 1934 *c*). The virus of feline agranulocytosis is not related (Lawrence *et al*, 1943).

W VIRUS

Gay and Holden (1932–3, 1933 *a, b*) obtained a very similar virus from the same fatal case They were able to transmit the infection to rabbits by intracerebral injection The animals die after 4 to 6 days with fever, salivation, tremors, and

paresis of the lower extremities Intranuclear inclusions are readily found, and in Ammon's horn involve nearly every cell. After intradermal injection, the results are similar to those obtained with B virus, an ascending paresis following a local hemorrhagic and necrotic lesion

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CHAPTER CII

INFECTIVE HEPATITIS

CLINICAL FEATURES

Adults.

William Stokes (1839) of Dublin, is credited with having recognized that jaundice was due to a mild form of hepatitis.

After an incubation period of about 30 days (see p. 1169) the disease commences with a history of malaise, anorexia, weakness, upper abdominal discomfort, bodily aches and pains, vomiting, and headache with low fever up to 103° F. usually lasting for about 5 to 7 days, or at times as long as 3 weeks. This is followed by the stage of jaundice of varying intensity, necessitating hospital care for a fortnight to 3 months, depending on the severity of the attack In mild cases, jaundice disappears in 2 weeks, and in the severest after 8 weeks In addition to the well known description of Cameron (1943) the earlier accounts of Blumer (1923), Pickels (1936–1942), and Cullinan (1939) should be consulted.

A summary of the signs and symptoms observed in 260 cases of hepatitis with jaundice has been provided by Zimmerman, Lowry, Uyeyama, and Reiser (1947). Over half the cases complained of symptoms of anorexia, dark urine, malaise, nausea, ocular pain, chilly sensations, headache, fever, and epigastric discomfort, and the remainder of vomiting, heartburn, constipation, pruritus, alternate constipation and diarrhea, respiratory discomfort, chills, diarrhea, clay-colored stools, and dysuria.

Clinical signs elicited in over half these cases were jaundice affecting both skin and sclera, usually tenderness of epigastric region, enlarged liver and adenopathy of generalized or cervical distribution. Less than 50 per cent. displayed splenomegaly, bradycardia, fever, rash, and herpes labialis During the icteric stage of the illness, Kirk (1947) reported that mild mental depression was not uncommon but during prolonged icterus confusional states were prone to ensue Cerebral manifestations, persistent vomiting and ascites are prognostic signs of evil significance according to Lucké (1944)

Children.

The disease tends to pursue a milder course Anorexia, vomiting, diarrhea, and abdominal discomfort are of shorter duration than in adults and severe hepatitis with prolonged icterus and long convalescence are unusual. (See Cockayne, 1912, Cullinan, 1939, Evans, 1942, Ford, 1943, Lisnev, 1944, Horstmann, Havens, and Deutsch, 1947.)

In England, biochemical tests on child contacts showed that infection may occur in the complete absence of icterus (Pollock, 1945 *b*) Similar observations were made at New Haven, by Horstmann, Havens, and Deutsch (1947) who commented upon the mildness of symptoms, their short duration, and the difficulty in persuading children to remain in bed.

Neurological complications Four varieties of nervous lesions have been described, all of which were believed to be due to the effects of chemical toxic absorption from the gut, resulting from liver dysfunction The first of these were

general in character, namely, coma, delirium, convulsions, and incontinence Secondly, there were striatal and pyramidal lesions characterized by increased tendon reflexes, clonus, and muscular rigidity with extensor plantar responses, occasionally with choreiform movements and parkinsonian tremor. The third group showed massive hemorrhages into caudate nucleus and subdural hematomata, and the last, peripheral neuritis (Stokes, Owen, and Holmes, 1945)

Mortality Whether cases be epidemic or sporadic the mortality is low. Lucké (1944) states that the rate in the United States Army outbreak of 1942 was 0 44 per cent For the Civil War, World War I, the German Navy (between the years 1919 and 1929), the Finnish outbreak of 1933, 1936, and the later Swedish epidemics the mortality ranged from 0 13 to 0 44 per cent.

PATHOLOGY

Clinical Pathology.

A good account is given by Zimmerman, Lowry, Uyeyama, and Reiser (1947).

Icteric index. Case values ranging from as low as 10 to as high as 180 have been recorded, the former case took about 7 days to return to normal and the latter 58 days No plateau was observed, that is, the peak was quickly reached and thereafter recession was prompt

Blood count. There was a tendency toward reversal of the lymphocyte leukocyte ratio and a slight eosinophilia (see also Havens and Marck, 1946).

Serum proteins. Using the copper sulfate method 81 severe cases showed serum [illegible] rsal of the [illegible] gh (Westergren) rate of 10 to 30 mm per hour encountered in a case of 4 to 8 weeks' dura- [illegible]

[illegible] flocculation, the hippuric acid excretion, the galactose tolerance, the bromsulfthalein excretion, and the thymol turbidity reaction (see Pollock, 1945 *a*, Dick, 1945, Kunkel and Hoagland, 1946, Makari, 1946).

In children increa[illegible] ported by Rapoport [illegible] of jaundice is claimec [illegible] protein reactions see Bjørneboe (1946 *a*). *X-ray and gastroscopic* examination of cases showed that acute gastritis was an early lesion of the disease (Havens, Kushlan, and Green, 1947)

Morbid histology of liver in biopsy specimens.

The early investigations of Iverson and Roholm (1939) showed that a small cylindrical biopsy specimen of the right lobe of the liver could be extracted with the aid of a 2 mm bore cannula passed transpleurally under local anesthesia. The operation is by [illegible] rhage and is, th [illegible]

The above s[illegible] Sherlock (1945) who obtained 61 such biopsy specimens from 14 cases of infective hepatitis, 35 of arsenotherapy jaundice, and 7 of serum jaundice No histological differential criteria could be recognized between any of the above conditions and

Dible, McMichael, and Sherlock (1943) are quoted thus: "A hepatic inflammation of varying intensity and distribution is common to them all, and, therefore, for purposes of pathological description, we may disregard this etiological grouping and consider the findings as a whole. The changes in the liver are related to the severity and duration of the disease. For purposes of comparison we have dated the condition from the onset of jaundice although there is always a variable period of diverse prodromal symptoms." These authors give the following account of the histology

Broadly speaking, the picture is one of hepatic cell necrosis and autolysis, associated with leukocytic and histiocytic reaction and infiltration The centers of the lobules show the first of these changes most markedly, and the portal tracts the greatest cellular infiltration In certain cases, which seem to be either those which are mild *from the beginning or in which the lesion is retrogressing, the periportal* cell accumulations predominate in the picture, in contradistinction to the more severe cases in which hepatic cell degeneration is more pronounced and the histiocytic and leukocytic infiltration more widespread For descriptive purposes we call the first of these the "zonal" and the second the "diffuse" type of change, but it must be understood that there are no good reasons for regarding these differences as fundamental, and that an intermediate picture occurs which has been denominated a "mixed" lesion. Finally, there are those cases in which the lesion is well on

[illegible] necrosis, nodular
[illegible] very significant
[illegible] the pathological

picture. Several of the cases seem to have been verging on acute liver necrosis—12 out of 56 cases showed histological damage of more than 50 per cent. of liver cells —yet the normal course is to complete recovery. Fatal or permanent damage is rare. Lucké (1944) also mentioned that there was no evidence of residual damage following recovery from infective hepatitis.

The frequency with which a periportal "cirrhosis-like" picture is encountered leaves little doubt that it constitutes one pathological form of acute hepatitis. Roholm and Iversen (1939) also noted this appearance, but were uncertain whether it represented a long-standing hepatitis with jaundice resulting from an acute exacerbation. That acute and subacute necrosis and cirrhosis could follow epidemic hepatitis has been recognized previously (Bergstrand, 1930, Cullinan, 1936, Krarup and Roholm, 1941).

Jaundice persisting over 2 weeks is more likely to be due to a zonal lesion Diffuse hepatitis usually heals completely and rapidly. When the disease runs a longer course some residual fibrosis in the portal zones may still be present after apparent clinical cure. There is no evidence that there is a form of jaundice due to duodenal catarrh and obstruction of the common bile duct by mucus (see also van Rooyen and Gordon, 1942).

Mechanism of icterus production.

Dible, McMichael, and Sherlock (1943) have provided the following explanation which we quote verbatim "The mechanism of production of icterus is a matter of some interest The disruption of the liver-cell columns with their inter-cellular bile canaliculi may create a form of obstruction to bile flow The isolation of liver cells may also lead to the excretion of bile into the surrounding tissue spaces It is also obvious that the degree of damage may be such that the surviving liver cells are quantitatively inadequate to excrete the bile pigment brought in the blood stream Histologically, pigmented cells are seen, especially at the center of the lobule Some of these are obviously necrotic, while others have lost their glycogen The latter may accumulate bile because they are unable to excrete it, while the former become pigmented in consequence of their death The affinity of dead tissue for bile is a common pathological phenomenon. Further out in the lobule

we meet with "bile thrombi," the finest intercellular canaliculi being filled
has obviously failed to escape There is an absenc
of the bile ducts, sugge

Appearances in fatal cases.

A full account has been given by Lucké (1944) and essential features ar
follows Lesions found in the liver resembled those typical of yellow atr
accompanied by extensive destruction of parenchyma, with skeletal remnant
isolated lobules outlined by small proliferating bile ducts The framework
sinusoids of damaged cells remained intact Hyperplasia of surviving cells
coarse nodular formation and subsequent degeneration due to ischemia, were
bladder was normal. *Regional lymph nodes* were edematous
to cellular proliferation The

1946).

GEOGRAPHICAL DISTRIBU... NT

For over a century this disease has figured conspicuously in European mil
entity under a multiplicity of names suc
jaundice of campaigns, jau
)19, Buchbir
1920, and Dietrich, 1942) erican Civil
(1861–65) according to Woodward (1863) Likewise appearance a
time of the Franco-Prussian War (1870–1) (Seggel, 1872, Hubener, 1917), and
South African War (Willcox, 1916) Cases occurring at Gallipoli and throug
the Middle East during 1914–1919 were described by Hunter (1922).

In the years between the first and second World Wars, periodic spo
outbreaks of infective hepatitis or catarrhal jaundice were recognized in
primarily affecting schoolchildren and a
were prov
War II s
rrival of U
l these abso
of the M
an
pr

Egypt, Greece,
Palestine, Eritrea, and Malta (see van Rooyen and
Damodaran and Hartfall, 1944)
Africa and the landing of British

(Sawyer *et al*, 1944
Hanlon, 1946) The "boom" or "oil" towns in the

Alberta, Canada, were attacked in 1941 and 1942 (Somerville and Clark, 1944). Probably thousands of cases occurred in Great Britain, Stocks (1944) alone reported *4,100 cases from January, 1942, to June, 1943, and many others* were reported by Follows (1940), Ford (1943), Edwards (1943), and Cookson (1944). Lisney (1944) reported 1,062 cases in Leicestershire, nearly half of which affected children of 5 to 10 years of age. In the Mariana Islands of the Pacific Ocean, a division of American Marines who entered combat on June 15th, 1944, first on Saipan and later Tinian, suffered [illegible] l of Ceylon was affected (Jayaw [illegible]).

On [illegible] the Scandinavian countries by Wickstrom (1940), Selander (1939), and Hallgren (1943), from Denmark by Bjørneboe (1946 *a*, *b*); and 600 cases from the German-Russian fighting front by Jacobi, Kreyenberg, and Dörschel (1943). For further data on the incidence of infection in the German-Italian Armies see Dietrich (1942), Gutzeit (1942), Siegmund (1942), and von Bormann *et al* (1943). Leningrad was affected in 1942 and 1943 (Viskovsky, 1944).

The above references describe reported outbreaks over a wide area of the globe, but it can be assumed that during the war years, 1941 to 1944, infective hepatitis was world-wide in distribution. With the cessation of hostilities and the dispersal of troop concentrations its incidence has declined.

EPIDEMIOLOGICAL CHARACTERS

Within and without the armed forces, World War II provided unique opportunities for study of the relative susceptibility of different social, economic, and racial strata of individuals to infective hepatitis Much has been written but relatively little is understood.

Racial susceptibility.

In the armed forces, the disease appeared to affect young soldiers, principally those of European stock, on active service abroad, the highest incidence probably was recorded in the Mediterranean theater of operations In Egypt, whereas United Kingdom, New Zealand, Australian, American, and certain South African soldiers were readily infected, there was reason to believe that the case rate was lower among troops who hailed from other parts of the British Commonwealth. In December, 1942, some 15 per 1,000 contracted jaundice in General Montgomery's famous Desert Eighth Army (Spooner, 1944) and during the earlier month of October 35 2 per 1000 suffered from it in the Second New Zealand Expeditionary Force, but Kirk (1947) commented that the 28th Maori Battalion and the Indian troops who served with this Army were only very lightly affected Likewise although the condition was prevalent among British personnel of General Headquarters and other military base establishments, centered around the outskirts of Cairo, there was a conspicuous absence of cases among local adult Egyptians (van Rooyen and Kirk, 1946) Similar observations were made regarding the disparity in case incidence between the military and civilian populations of Palestine (Cameron, 1943) This feature was still more obvious in the island garrison of Malta where the attack rate was 13 7 per 1000 in British soldiers, 0 24 per 1000 among locally enlisted Maltese and apparently no epidemic was noted among Maltese civilians (Dixon, 1944). Supplementary evidence to support these claims was furnished by Kligler, Btesh, and Koch (1944) who pointed out that each successive group of newly arrived European Jewish immigrants to Palestine, especially children, was vulnerable to attack. In Ceylon, relatively few local Ceylonese troops were admitted to hospital [illegible] French Arab troops serving in Italy sh [illegible] o Gauld (1946) but the Brazilian [illegible] autumn and winter of 1944–45 seemed to escape

Social class selection.

It was noticed in Egypt that British Army Officers, as a class, were about 4.7 *times* as susceptible to attack as other ranks (Spooner, 1944). Similar epidemiological field studies in N Africa and Italy among American Forces by Gauld (1946) revealed that the officer : other rank, attack rate ratio, varied in different types of [illegible]

Effect of seasoning.

Gauld (1946) showed that for a single period in a total of 3 regiments in troops who had lived through a previous epidemic the attack rate was 39 7 per 1000 against 108 7 per 1000 for reinforcements. During 1944–5 in 6 divisions of General Eisenhower's Fifth Army the highest rates were returned from units receiving the largest reinforcements.

Seasonal incidence.

In Egypt, the months of August and September showed the greatest number of cases (van Rooyen and Kirk, 1946), but in America and elsewhere cases may occur throughout the whole year (Havens, 1946 *a*) and the reasons for such variation is not known. It is not improbable that if sanitary conditions are very bad at any place the disease may break out

Incubation period.

Under natural conditions, the interval between the calculated day of exposure to infection and onset of jaundice has been estimated to be a minimum of 32 days by Cameron (1943) But if the date of onset of symptoms prior to icterus is accepted then the period may be taken as 20 to 40 days with an average of 28. The period for the disease experimentally induced by feeding icterogenic serum or heavily infected feces, may range from 16 to 85 days and is usually about 25 days, according to Havens (1946 *a*) MacCallum and Bradley (1944) found the time to be 27–31 days. Wide variation in the incubation period encountered by *different* workers hints at the existence of multiple strains of virus, varying in virulence Gradations in individual susceptibility of different batches of volunteers to infection may also be an important factor

In an interesting experiment Francis, Frisch, and Quilligan (1946) showed that blood obtained from an incipient case of infective hepatitis and inoculated intravenously in a volunteer elicited the first symptom in 11 days and jaundice in 21. They were also able to demonstrate that the virus of infective hepatitis was present in blood of the recipient on the 8th day after transfusion, *namely*, 3 days before the first symptom of illness, as well as 3 days after it and 13 days before jaundice appeared In 4 subjects, the incubation period varied from 42 to 47 days and likewise the clinical severity Francis and his coworkers point out the difficulty of attempting to classify infective hepatitis on the basis of incubation period after parenteral injection of icterogenic serum. Furthermore, they *are not convinced* that the dose of virus is totally unrelated to the clinical severity of the resulting disease as popularly thought Finally, most important of all, they point out that the occurrence of virus in blood well before the onset of infective hepatitis, may result in its transmission by blood products derived from individuals in whom the presence of virus would be entirely unsuspected

Subclinical attacks.

Adults. It is possible that missed cases of the disease may play an integral part, in the dissemination of infection Clinical observations in the isolated garrison of Malta by Dixon (1944) and in N. Africa by Hunt (1944), have emphasized this aspect.

ETIOLOGY

According to Migne (1844), the contagious character of jaundice is said to have been referred to in a letter from Pope Zacharias A.D. 741–752, to Boniface (Wynfrith) the apostle of Germany, concerning cases among Germans.

Little is known of the causal agent of infective hepatitis although it is generally accepted to be a virus. Thus, failure to isolate a specific pathogenic cultivable bacterium from blood, feces, or urine, plus the fact that the condition can be transmitted, in man alone, by parenteral inoculation of bacteriologically sterile filtered serum or ingestion of feces, amount to the sum total of evidence upon which the icterogenic agent has been classified as a virus For a comprehensive account of human experimental studies see Paul, Havens, Sabin, and Philip (1945).

TABLE 40

REPRODUCTION OF INFECTIVE HEPATITIS WITH JAUNDICE IN HUMAN SUBJECTS

Inoculum	*Route*	*Numbers of Volunteers*		*Incubation Period, Days*	*Reference*
		Tested	*Jaundiced*		
Duodenal fluid	oral	4	1	28	Voegt (1942)
Urine	oral	?	1	?	Voegt (1942)
Blood	oral	?	1	?	Voegt (1942)
Feces	oral	3	2	20–22	Havens, Ward, Drill, and Paul (1944)
Serum	oral	5	4	23–34	Havens (1945 *a, b*)
Feces	oral	3	2	16–17	Havens (1946 *a*)
Feces	oral	18	7	17–28	Findlay and Willcox (1945)
Urine	oral	6	3	?	Findlay and Willcox (1945)
Feces	oral	72	31	20–30	Neefe, Stokes, and Reinhold (1945)
Serum	oral	3	2	26–33	Neefe and Stokes (1945)
Feces	naso-pharyngeal	26	3	27–31	MacCallum and Bradley (1944)
Serum	sub-cutaneous	?	1	19	Voegt (1942)
Blood serum	intra-muscular	6	6	30+	Cameron (1943)
Serum	sub-cutaneous	21	4	85–106	Oliphant (1944)
Serum	sub-cutaneous	6	3	64–92	MacCallum and Bradley (1944)
Serum	sub-cutaneous	17	9	20–31	Havens (1945 *a, b*)
Serum	intra-venous and sub-cutaneous	9	5	21–47	Francis, Frisch, and Quilligan (1946)

Distribution of virus in man.

Virus is present in the serum and whole blood of established jaundiced cases of the natural disease (Cameron, 1943), and in feces (MacCallum and Bradley, 1944; Havens, Paul, van Rooyen *et al.*, 1945). Volunteers fed with feces showed virus in their stools on the 5th day of illness prior to the onset of jaundice, but by the 25th day virus had disappeared from stools Pooled stools from convalescent cases on the 25th and 29th days were noninfective to volunteers Similar tests with serum [illegible]

[illegible] tained virus A summary of human experiments appears in Table 40

Properties of the virus.

Filtrability The agent has been shown to pass the Mandler filter under 9 lbs pressure per square inch (Francis, Frisch, and Quilligan, 1946), the Chamberland No 2 filter (Havens, 1945 *a*), and Seitz filter by Neefe and Stokes (1945).

Heat It is unaffected by exposure to 56° C for 30 minutes (Havens, 1945 *a*).

Cold. Like other viruses, the agent can be preserved at low temperatures in CO_2 ice (at −70° C.) for 8 months (Havens, Ward, Drill, and Paul, 1944).

Chemicals. In contaminated water, one part per million residual chlorine failed to inactivate the virus (Neefe, Stokes, Baty, and Reinhold, 1945), but if water was treated with coagulation, filtration, and settling, then inactivation occurred (Neefe *et al*, 1947)

Cultivation The virus has not been cultivated in the embryonated egg, and claims to have transmitted it to animals have lacked confirmation, likewise, reports concerning the presence of elementary bodies in infected tissues are lacking.

Therapeutic effect on rheumatoid arthritis.

MacCallum and Bradley (1944), claimed that cases of this disease were improved by an artificially induced attack of infective hepatitis Relapses of arthritis have been reported by Rennie and Fraser (1946)

Mode of Spread

Infection is almost certainly conveyed by the alimentary tract (Willcox, 1916). Blumer (1923) also suggested that infectious jaundice in the United States may be spread by the oral route.

Water. In the light of modern knowledge, an early description of a unique outbreak of infective hepatitis which affected students at Mount Allison University in Sackville, New Brunswick, Canada, in December, 1930, and January, 1931, which was investigated with great thoroughness by Fraser (1931) makes interesting reading Fraser (1931) studied 173 cases of "catarrhal" jaundice which he found was preceded by an [illegible]
be infectious b [illegible]
was excluded [illegible]
water was bla [illegible]
(1931) failed to isolate any pathogenic bacterium or leptospira from the suspected water and that the high level of *Salmonella schottmulleri* agglutinins in human sera [illegible] sanitation. For details of a recent case of [illegible] idary invasion with *Salmonella*, see Havens [illegible], N. Sweden, Hallgren (1943) reported an epidemic which appeared to be of water-borne origin. Another outbreak of water-borne origin has been reported by Neefe and Stokes (1945) at a summer camp for children Apparently the girls' drinking water well aroused suspicion because it was

Subclinical attacks.

Adults. It is possible that missed cases of the disease may play an integral part, in the dissemination of infection. Clinical observations in the isolated garrison of Malta by Dixon (1944) and in N. Africa by Hunt (1944), have emphasized this aspect.

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Serum	intra-venous and sub-cutaneous	9	5	21–47	Francis, Frisch, and Quilligan (1946)

Zealand soldiers From the results of statistical analysis, by comparing the incidence of cases in front and rear line troops and after eliminating food, water, mosquitoes, and other obvious factors, Kirk (1945) concluded that the outbreak among the New Zealanders was fly-borne in character. The ground held by NZEF had been recaptured from the enemy, it was grossly polluted with feces and imperfectly buried corpses, flies were innumerable and sanitation extremely bad. During the period under review on the Alamein line at the time of the Battle of Ruwyeisat Ridge, fought on July 14th, 1942, the wind blew from the enemy lines across "no man's land" on to the defended front, carrying with it swarms of flies and the odor of decomposing bodies, a scene repugnant to eye and nostril. So bad were the flies that it was impossible to protect food from them and many preferred to eat after dark and before dawn. Another account of the fly menace in the Western Desert was provided by Richmond and Gear (1945) who wrote that after the retreat from Gazala to El Alamein, flies assumed the proportions of a plague

Urine.

Although Findlay and Willcox (1945) successfully infected volunteers by feeding urine, the work has not been repeated (Paul and Havens, 1946)

Rats.

These have been suspected to play a part in the dissemination of infection at Leningrad by Viskovsky (1944)

Summary of evidence.

Infective hepatitis is a disease of communal living and communal feeding During wartime it flourishes in camps and armies, and in peacetime in civilian institutions, where amenities for the maintenance of personal hygiene, ablution, laundry, and cleaning services are restricted

Immunity Phenomena in Infective Hepatitis

It is not improbable that immunity to this disease may be more widespread among nations and communities than generally supposed, and that such resistance may frequently result from unrecognized subclinical attacks Thus, Stokes and Neefe (1945) showed that human gamma globulin derived from healthy blood donors contained neutralizing antibodies which, when administered to infected human volunteers in the incubation period, was capable of preventing or attenuating the onset of infective hepatitis (see also Gellis *et al*, 1945 *a*, *b*, and Havens and Paul, 1945) It has also been shown by Neefe, Stokes, and Gellis (1945) and Havens (1946 *d*) that one attack of experimentally induced infective hepatitis confers immunity against a second attack of the same agent possibly for many months

Second attacks.

During large epidemics, these are relatively uncommon Experimental work (Havens, 1946 *d*) on man also suggests that a single attack of infective hepatitis provides immunity against the same strain of virus, but Havens (1946 *a*) has declined to answer the question whether such immunity conferred resistance toward multiple strains of virus Chronic hepatitis in the Mediterranean area has been reported by Barker, Capps, and Allen (1945), the etiology of which is obscure.

Cross immunity with serum jaundice.

Evidence is conflicting, but it is probable that no cross immunity exists between the 2 diseases One attack of serum jaundice does not protect against subsequent attacks of infective hepatitis according to Havens (1945 *b*) but Oliphant (1944)

surrounded at a distance of *75 to 180 feet by 4 cesspools which received* sewage from the girls' cabins. Five volunteers each drank 7000 c.c. of water from this well and 61 to 78 days later all 5 men developed symptoms of hepatic dysfunction.

Food The source of an explosive outbreak occurring at a university students' club was narrowed down to infection acquired from food taken on one or two days (Read *et al.*, 1946).

Milk. Milk has been blamed for an outbreak of 10 cases affecting a group of 144 persons living in 26 homes at Georgia who received milk from the same dairy.

Droplet infection.

Epidemiological studies by workers in Britain (Edwards, 1943), in the Marianas (Getty, 1946), and studies on troops who had returned to Britain from Sicily (McFarlan, 1945) suggest that the disease is disseminated by close contact droplet infection These observations were not confirmed by Spooner (1944) who studied the major 1942 epidemic of infective hepatitis affecting British troops in Egypt, Palestine, and the western desert. Spooner (1944) stated that 2 observations were inconsistent with a droplet spread. One was that there was no tendency for more than one case to occur in a vehicle crew stationed in a lonely desert outpost, and when a second case did develop it usually happened within a fortnight of the first The other objection was that the disease failed to spread in prisoner of war camps, where infected new arrivals did not appear to infect their comrades and pen mates who had been taken earlier. To this, the authors would add that despite the bomb damage and gross post-war domestic overcrowding prevailing in Great Britain today, the incidence of infective hepatitis has decreased and not increased as may have been anticipated, if droplet infection is the customary method of spread.

To date, all efforts in the United States to demonstrate the virus of infective hepatitis in nasopharyngeal washings have failed (Paul and Havens, 1946).

Fecal contamination.

Infective hepatitis has so frequently been reproduced in human volunteers (see Table 40) by feeding infected stools, that it requires little imagination to conjecture how the disease could spread in countries such as Egypt where the general standard of sanitation is not high and even cholera may occur, as it did in 1947 The resistance of infective hepatitis virus to heat, drying, and such antiseptics as chlorine, reveal the potentialities of this virus as an agent capable of causing massive infection among insanitary communities.

van Rooyen would like to quote 5 interesting cases brought to his notice by fellow officers in 1942 and 1943, where the circumstances aroused suspicion that the disease was one transmitted by fecal contamination and fomites The first was that of a young soldier suffering from severe gunshot injuries to the spine, with resulting paraplegia and incontinence of urine and feces This man was sponged daily and tended by 2 full-time army nurses Shortly after admission he developed jaundice and a month later both nurses likewise contracted infection. There were no other cases among the nursing staff at the time

The second instance occurred when 2 men attached to a large base depot quartermasters store suddenly developed jaundice in the month of June, 1943, at a time when no other cases existed in the unit Inquiries revealed that both men had been occupied in sorting out and handling the soiled battle dress clothing of New Zealand soldiers who had been killed in action

Flies Kirk (1947), whose experience of this disease in the field is unsurpassed, studied 1,500 clinical cases admitted to the wards of the First New Zealand General Hospital between September and December, 1942 He also conducted first hand epidemiological studies in the Western Desert when serving with the Second New Zealand Expeditionary Force attached to the Eighth Army between July, 1942, and January, 1943, when 2,095 cases occurred among some 15,000 front line New

CHAPTER CIII

HOMOLOGOUS SERUM JAUNDICE

An icterogenic agent, presumably a virus, is now recognized to occur in the serum of a proportion of apparently healthy individuals The parenteral administration of blood, plasma, or serum from such persons by syringe and needle, whether for the prevention of measles, mumps, sandfly fever, for blood transfusion, or through the

responsible for much

with those of infective hepatitis Moreover, the properties of these two etiological agents are so very similar that serum jaundice virus may be a variant form of infective hepatitis It is also important to remember that some examples of apparent "serum jaundice" may represent artificially induced infective hepatitis, although all serum jaundice may not be due to this cause (Paul and Havens, 1946) Both conditions are species-specific for man, and, notwithstanding the generosity of volunteers in America, Britain, and Europe who have cheerfully submitted to experimentation at the risk of serious liver injury and death, wide gaps exist in the state of medical knowledge of the pathogenicity of these agents

Clinical Features

The signs and symptoms of serum jaundice are indistinguishable from those of infective hepatitis (see Ch. CII, also Memorandum, 1943) Likewise, no clinical distinction is possible between postarsenical jaundice and infective hepatitis (Mitchell, 1943).

Pathological Changes

Changes and lesions are identical with those of infective hepatitis, see p 1165

History of Outbreaks

The disease first attracted attention in Great Britain in 1937 when 43 cases with 9 deaths occurred among a group of 123 children who received 2 batches of *antimeasles convalescent serum* (McNalty, 1938, and Memorandum, 1943). An outbreak at a mental institution under similar circumstances was reported by Propert (1938).

Postyellow fever vaccine jaundice.

In Britain, Findlay and MacCallum (1937–38) and Findlay (1940), first reported cases of jaundice contracted at intervals varying from 2 to 7 months after inoculation with tissue cultured yellow fever virus vaccine containing normal human

was reported from Brazil by Soper and Smith

ld War II when troops were mobilized and tlantic preparatory to service overseas, many thousands were inoculated against yellow fever in America, and certain batches of vaccine proved to be icterogenic. In the year 1942 an extensive outbreak was reported in the United States Army by Turner, Snavely, Grossman, and Buchanan (1944). A series of 10,284 cases, 31 of which were fatal, was investigated by Sawyer, Meyer, Eaton, Bauer, Putnam, and Schwentker (1944 *a*, *b*). The latter concluded that the cases were due to the introduction of an unknown icterus-producing filtrable virus in the blood of apparently normal blood donors whose serum was

reported the opposite effect. Conclusions have been drawn from the results of tests employing small numbers of human volunteers and are thus unsatisfactory.

Prophylactic value of gamma globulin.

If given during the incubation period of infective hepatitis it has been reported to modify the subsequent course of events, according to Stokes and Neefe (1945), Gellis *et al.* (1945 *b*), and Havens and Paul (1945).

Complement fixation reactions.

Attempts to elucidate specific reactions have been unsuccessful (Miles, 1946)

Attempts to Infect Animals

Pigs are said to have been infected with the virus of infective hepatitis in Denmark by Andersen and Tulinius (1938) but van Rooyen and Gordon (1942) failed to infect pigs.

Birds. Canaries are thought to be susceptible by Dresel, Meding, and Weineck (1943), and Herzberg (1943–4), but resistant according to Hoyle (1943).

Guinea-pigs. Verlinde *(1946) has claimed positive results.*

Rats. MacCallum and Miles (1946) produced lesions with blood and feces from cases of infective hepatitis in animals fed on a deficient diet.

Chimpanzees. Havens and Ward (1945) fed 6 of these animals with material known to contain infective hepatitis virus. The serum bilirubin, bromsulfthalein and cephalin flocculation tests were all negative and it was concluded that the animals were refractory to inoculation.

Monkeys Lasiopyga griseoviridis or *Cercopithecus aethiops* monkeys and Abyssinian baboons, *Papio hamadryas* were repeatedly inoculated with blood, urine, feces, and stomach washings by different routes but without success. Monkeys were also fed by stomach tube with several grams of desiccated serum from infective hepatitis cases mixed with powdered glass, but with no effect (van Rooyen and Gordon, 1942, van Rooyen and Kirk, 1946)

Mice, gerbilles (Gerbillus gerbillus) and *Gerbillus pyramidum, desert rats (Jaculus jaculus), pigeons,* and *kittens* were inoculated with blood from early and late cases of infective hepatitis. Liver tissue was inoculated for several blind passages, but with completely negative results throughout (van Rooyen and Gordon, 1942).

Chick embryos According to German workers a transmissible agent has been propagated in the chorio-allantoic membrane of chicken embryos (Siede and Meding, 1941, Dresel, Meding, and Weineck, 1943)

Elementary bodies were also said to be found in egg membranes and *in patients* (Essen and Lembke, 1944, Ruziczka, 1943) These claims have not been confirmed by Hoyle (1943) and others. Essen and Lembke (1944) claim to have found polyhedral-shaped elementary bodies, using the electron microscope.

Serum jaundice at a diabetic clinic.

Sixty-three cases with 4 deaths among elderly diabetics over 55 years of age were reported by Droller (1945) at a clinic in Sheffield, England. Here syringes were not sterilized by boiling but subjected to the worthless procedure of washing in sterile distilled water and storing in spirit.

Properties of Virus

Filtrability. The agent traverses the Seitz asbestos disk filter without difficulty (Findlay, MacCallum, and Murgatroyd, 1939, Sawyer *et al.*, 1944 *b*).

Heat It withstands heating at 56° C. for 30 minutes (Sawyer *et al*, 1944 *b*).

Cold. The virus can be preserved in carbon dioxide ice ... er *et al*, 1944 *b*) ... phenol and ether for ... morandum, 1943).

Inactivation by ultraviolet light.

According to Oliphant and Hollaender (1946), a strain of serum jaundice virus contained in pooled serum derived from an English blood bank was inactivated by ultraviolet light irradiation 16.0 ml. of icterogenic serum was placed in a quartz glass flask, which was suspended and rotated between eight 8-watt low pressure mercury vapor lamps, from which 80 per cent of the radiation was at 2,537 Å Samples of serum were withdrawn after 1, 6, and 30 minutes, respectively, and injected subcutaneously in 12 volunteers One of those receiving serum after irradiation for 6 minutes, developed jaundice 84 days later, but the remainder was unaffected throughout the period of observation for 161 days. Simultaneously, 12 controls were inoculated with untreated serum and of these 4 developed jaundice.

If satisfactory irradiation apparatus could be devised for effectively treating large volumes of serum, this work would acquire considerable importance, and the value of the method may be put to the practical test.

Attempts at cultivation and animal infection.

All efforts to infect chicken embryos with blood, filtered duodenal contents, ...

Human transmission experiments.

Following parenteral inoculation of serum jaundice virus the *incubation period* had been stated to be 72 to 132 days (Paul, Havens, Sabin, and Philip, 1945), 59 to 129 days (MacCallum and Bauer, 1944), 45 to 150 days (Spurling, Shone, and Vaughan, 1946), and 44 to 167 days (Grossman, Stewart, and Stokes, 1945) After yellow fever vaccine, the period has been estimated to be 60–150 days (Sawyer *et al.*, 1944 *b*) and 2 to 7 months by Findlay and MacCallum (1937, 1938)

Studies on postarsenotherapy jaundice have shown the incubation period to be 62 to 157 days (MacCallum, 1945) and 62 to 157 days (Howells and Kerr, 1946).

Distribution of Virus in Man

The virus of serum jaundice has been shown to be present in the peripheral blood of one volunteer 34 days after inoculation and 60 days before this man developed jaundice (Paul, Havens, Sabin, and Philip, 1945) The disease has also been transmitted by feeding with infected serum (MacCallum and Bauer, 1944), but when feces and nasopharyngeal washings from cases of postarsenical jaundice were fed, MacCallum (1945) failed to do so. Neefe, Stokes, and Reinhold (1945) fed 19 volunteers with 3 pools of feces from cases of serum jaundice gathered

incorporated in the manufacture of yellow fever vaccine. It was, therefore, recommended that serum-free yellow fever vaccine be prepared in future Other interesting points were that there were few, if any, secondary cases, and no evidence of the disease having been contracted from civilians or troops having infected civilians

(For jaundice following use of human *antimumps serum* administered to troops in England, see Beeson, Chesney, and McFarlan, 1944)

Jaundice following administration of human *anti-sandfly fever serum* has been reported from as far distant as the Black Sea Coast of Russia by Sergiev *et al* (1940).

Posttransfusion jaundice.

With the start of active warfare, many battle casualties and civilian air raid victims who received serum/plasma transfusions subsequently developed jaundice. Careful studies in Britain established many important facts. Thus, some pooled batches of serum and plasma were found to be more icterogenic than others and, of 1,054 patients transfused, 77 (7 3 per cent.) developed jaundice 45 to 150 days later (Spurling, Shone, and Vaughan, 1946, Bradley, 1946). The *pros* and *cons* of employing single donors for each recipient as opposed to use of pooled sera and plasma from multiple donors were also considered Loutit and Maunsell (1945) concluded that it would be safest to transfuse serum from individual to individual, but that small pools of serum and plasma gathered from not more than 10 persons be permitted The effect of dosage was also scrutinized and Loutit (1944) reported that 50 out of 54 patients could be infected with serum jaundice virus with doses of serum as small as 0 1 c.c. administered intradermally. MacCallum (1946) further stated that the size of the inoculum, within wide limits, had no effect upon the attack rate, incubation period, or severity of the illness.

Use of gamma globulin.

Disappointing results have been reported by Grossman, Stewart, and Stokes (1945) and Robinson *et al* (1946) Failure is not surprising because the incubation period of the disease is long and in all probability viremia is well established long before the onset of clinical jaundice (Paul and Havens, 1946).

Postarsenical jaundice.

The authors' own observations at certain venereal disease treatment centers, civilian and military alike, before and after the war, are in agreement with the comments of others, respecting the low standards of asepsis practiced at these institutions (Bigger, 1943, Beattie and Marshall, 1944) Even under wartime conditions of shortage of equipment, Salaman *et al* (1944) and Laird (1946) have shown that the disease is preventable by correct organization, discipline, education, and supervision of orderlies at clinics

The high incidence of jaundice among syphilitics treated with arsenic, prompted Peters *et al* (1945) to employ sulfur-coated amino acids in an effort to diminish the possible hepatotoxic action of arsenic, but without marked benefit In a memorandum (1945) it was concluded that late hepatitis after arsphenamine, gold, and other therapies is an expression of homologous serum jaundice communicated by traces of blood transferred on syringes and needles from patient to patient

Postpenicillin jaundice.

The continuance of jaundice at certain venereal disease treatment centers despite the advent of penicillin, strongly suggests that the major cause of jaundice past and present at these units has been faulty sterilization technique. (See Darmady and Hardwick, 1945, Howells and Kerr, 1946, Hughes, 1946, and Turner, 1946.)

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from 7 days before appearance of jaundice to 18 days after it, but with negative results Findlay and Martin (1943) claim to have transmitted serum jaundice (employing icterogenic yellow fever vaccine) with nasopharyngeal washings.

Cross Immunity with Infective Hepatitis

The evidence is both conflicting and scanty. According to Oliphant (1944), individuals who had recovered from postyellow fever vaccine serum jaundice were resistant to reinfection with the homologous virus, as well as a strain of infective hepatitis virus, but Havens (1945 *b*) found that one attack of serum jaundice did not produce immunity against infective hepatitis, and Gauld (1946) observed that American troops who had previously suffered from serum jaundice, were two and a half times more susceptible than others to natural infective hepatitis when exposed to infection in Italy.

Experiments performed on small numbers of human volunteers, unless elaborately controlled, are prone to lead to statistically unsound deductions We lack much fundamental information, such as the relationship of the dosage of virus to the development of both varieties of infection. Some contend as little as a pin prick can convey serum jaundice. Others are uncertain. The authors know of a patient who was unaffected after transfusion of a pint of blood from a donor who developed jaundice the next day. In short, we possess no serological or immunological measure whereby the relative susceptibility or otherwise of an experimentally inoculated subject can be determined so as to permit interpretation of negative test with certainty. The relationship of these 2 viruses is subject to much conjecture We do not know whether they are different, closely related, or identical It has been tentatively suggested that clinical serum jaundice may represent the artificial production of infective hepatitis The latter possibility excites curiosity for if eventually the 2 are proved to be identical, then infective hepatitis virus may well be described as one possessing the unique property of extreme susceptibility to artificially induced variation such as by merely altering the natural avenue of infection in the normal host.

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INDEX